



New Neonatal Porcine Diarrhea Syndrome in Denmark Characterization of the intestinal lesions and identification of the etiology

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New Neonatal Porcine Diarrhea Syndrome in Denmark

Characterization of the intestinal lesions and identification of the etiology

PhD Thesis by

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2014

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Preface and acknowledgements

This thesis concludes research performed entirely at the Section for Bacteriology, Pathology and Parasitology, National Veterinary Institute, Technical University of Denmark (DTU) in the period from August 2010 to November 2013. The PhD project was as a part of a larger project carried out in collaboration between the National Veterinary Institute, DTU, Pig Research Centre, Danish Agriculture and Food Council and HERD-Centre for Herd-oriented Education, Research and Development, University of Copenhagen. That large project aimed to investigate the etiology, diagnostics and treatment strategies of New Neonatal Porcine Diarrhea Syndrome (NNPDS) in Denmark and was supported by the Danish Ministry of Food, Agriculture and Fisheries.

The work described in this PhD thesis has been performed under supervision of Senior Scientist Tim Kåre Jensen and Professor Mette Boye from the Section for Bacteriology, Pathology and Parasitology at the National Veterinary Institute, DTU.

I would like to express my deepest gratitude to all the people that have contributed to this work in various ways during the period of my study. First of all I would like to thank my supervisors Tim Kåre Jensen and Mette Boye for their valuable and effective guiding in the research and writing process and continuous help and support in all stages of my study. Special thanks should be given to all the people involved in the NNPDS project for sharing their knowledge that added a great value to my graduate experience and for their consisting attention to my work. I sincerely wish to thank my co-authors for their scientific input to articles and manuscripts presented in this thesis, constructive feedback and fruitful discussions.

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Last but not least I would like to thank my family and friends for their emotional support, love and understanding and for not letting me forget that there is also life outside of the microscopy room.

I would like to dedicate this work to the memory of my mother Grazyna Jonach (1957-2011), who strongly supported and encouraged me to take on the challenge of a PhD graduation but was not given the chance to witness the successful realization of this thesis.

Beata Jonach, August 2014.

Abbreviations

AEEC: Attaching and effacing *Escherichia coli*

AIDA: Adhesin involved in diffuse adherence

CD: Crypt depth

C. difficile: *Clostridium difficile*

C. perfringens: *Clostridium perfringens*

DTU: Technical University of Denmark

EAEC: Enteroaggregative *Escherichia coli*

EAST 1: Enteroaggregative heat-stable enterotoxin 1

E. coli: *Escherichia coli*

EDTA: Ethylene diamine tetraacetic acid

E. durans: *Enterococcus durans*

E. hirae: *Enterococcus hirae*

Enterococcus spp.: *Enterococcus* species

EPEC: Enteropathogenic *Escherichia coli*

ETEC: Enterotoxigenic *Escherichia coli*

FISH: Fluorescence *in situ* hybridization

GALT: Gut associated lymphoid tissue

GI tract: Gastrointestinal tract

G0 phase: Gap zero phase

G1 phase: Gap one phase

G2 phase: Gap two phase

HE: Hematoxylin and eosin staining

HIER: Heat-induced antigen retrieval

HRP: Horseradish peroxidase

Ig: Immunoglobulins

IHC: Immunohistochemistry

LP: Lamina propria

M phase: Mitosis phase

MT: Mucosa thickness

NNPDS: New Neonatal Porcine Diarrhea Syndrome

PED: Porcine Epidemic Diarrhea

PI: Post-inoculation

PP: Post-partum

PCR: Polymerase chain reaction

RNA: ribonucleic acid

rRNA: Ribosomal RNA

SD: Standard deviation

S phase: Synthesis phase

TBS: Tris-buffered saline

TGE: Transmissible Gastroenteritis

VA: Villus atrophy

V/C ratio: Villus height/crypt depth ratio

VH: Villus height

Summary

In recent years, a new form of porcine neonatal diarrhea, termed 'New Neonatal Porcine Diarrhea Syndrome' (NNPDS) has been reported from several European countries including Denmark. This condition is characterized by watery to creamy, non-hemorrhagic diarrhea occurring during the first week of life, high morbidity and relatively low mortality and weak or no response to the antibiotic treatment. This diarrhea affects pig farms despite good management practice and routine sows vaccination. None of well-known enteric pathogens causing neonatal porcine diarrhea has so far been associated with this syndrome.

The main focus of the work presented in this PhD thesis was to characterize the morphological changes in the intestinal mucosa of the piglets affected by NNPDS in respect to elucidation of the pathological mechanism and etiology. This was based on systematically performed microscopic investigation of the intestinal tissue from the diarrheic and non-diarrheic piglets collected from four herds affected by NNPDS. This investigation included histopathological, morphometrical, immunohistochemical and fluorescence *in situ* hybridization examination of the intestinal mucosa. In the second part of the PhD project, an animal model of NNPDS has been established. This model can provide basis for future studies on the pathogenesis of this new syndrome.

The results of the histopathological investigation performed on 51 diarrheic and 50 non-diarrheic piglets showed that 63% of the diarrheic piglets developed villus atrophy of various degrees with concomitant crypt hyperplasia in the jejunum and ileum (Chapter 4.1). Villus atrophy is a common pathological feature seen in numerous infectious intestinal conditions and is associated with malabsorptive diarrhea due to insufficient absorption of water and nutrients from the small intestine. The morphometric study performed on the intestinal tissue showed that the diarrheic piglets had significantly shorter villi, deeper crypts, and thinner mucosa in the jejunum and ileum compared to the non-diarrheic piglets (Manuscript I). Reduction in villus height led to decrease in villus/crypt ratios which suggest an adverse effect on gut absorptive functions. Intestinal tissue samples from 24 representative piglets were subjected for immunohistochemical study on cell proliferation and apoptosis (Manuscript II). The results of this investigation showed that villus atrophy was associated with enlargement of the proliferative compartment in the crypts and that epithelial cell turnover was enhanced in the diarrheic piglets.

Potentially pathogenic bacteria such as *Escherichia coli*, *Enterococcus* spp., *Clostridium perfringens* and *Clostridium difficile* have been proposed to be involved in NNPDS. In order to elucidate their role in the pathogenesis of the syndrome, fluorescence *in situ* hybridization (FISH) with oligonucleotide probes targeting rRNA specific for these bacteria was applied on the formalin fixed, paraffin embedded intestinal tissue samples (Manuscript III). The results of this investigation showed that adherent *E. coli* and *Enterococcus* spp. were involved in NNPDS. These bacteria were present in 37% of the diarrheic piglets and were associated with villus atrophy and epithelial lesions in the small intestine. No clear association between the presence of *C. perfringens* and *C. difficile* and diarrhea was shown by FISH investigation.

In order to provide basis for further studies on the pathogenesis of the syndrome, an animal model of NNPDS has been established (Manuscript IV). Eleven litters of newborn piglets were orally challenged with intestinal tissue homogenate derived from four diarrheic field herds previously investigated in the project

and one healthy herd. Diarrhea was successfully reproduced in piglets from six litters and these piglets developed pathological lesions similar to those seen in the natural infection. Neither piglets challenged with the homogenate from the healthy herd, nor with the filtrate deprived of bacteria of three case homogenates developed diarrhea. This indicates that NNPDS is of a bacterial, rather than a viral nature. The course of experimentally induced diarrhea differed in severity of the symptoms and histological lesions depending on the inoculum used in the experiment, suggesting that herd-related factors may influence the clinical outcome of NNPDS.

Taken together, the work described in this thesis contributed to the knowledge on the pathogenesis of NNPDS, however the exact etiology of the syndrome has not been determined. Furthermore, this study has provided a reproducible model of NNPDS that can be the basis for future investigations on this emerging syndrome in newborn piglets.

Sammendrag (Danish summary)

I de seneste år, er en ny form for neonatal diarré hos grise, betegnet som 'Ny Spædgrisediarré Syndrom' (New Neonatal Porcine Diarrhea Syndrome/NNPDS) blevet rapporteret i flere europæiske lande, herunder Danmark. Syndromet er karakteriseret ved vandig til cremet, ikke hæmorrhagisk diarré inden for den første leveuge, høj sygelighed og relativ lav dødelighed og en svag eller ingen respons på behandling med antibiotika. Diarréen ses i besætninger trods godt management og rutinemæssig vaccination af søer. Ingen kendte tarmpatogener, som forårsager diarre hos smågrise, er hidtil associeret til dette syndrom.

Denne afhandling er bygget på forskellige undersøgelser med formålet, at karakterisere morfologiske forandringer i tarmslimhinden til bestemmelse af patologiske mekanismer og mulige årsager til NNPDS. Dette er baseret på systematisk udført mikroskopiske undersøgelser af tarmvæv fra grise med- og uden diarré, som var indsamlet fra fire besætninger ramt af NNPDS. Mikroskopien omfattede histopatologiske, morfometriske, immunhistokemiske og fluorescens *in situ* hybridiserings (FISH) undersøgelser. Den anden del af PhD projektet var fokuseret på etablering af en infektionsmodel af NNPDS, til at skabe grundlag for fremtidige studier af patogenesen af dette syndrom.

Resultaterne af den histopatologiske undersøgelse, udført på 51 smågrise med og 50 smågrise uden diarré, viste at 63% af diarrégrisene udviklede villusatrofi i forskellig grad med samtidig krypthyperplasi i jejunum og ileum (Kapitel 4.1.) Villusatrofi er et fælles patologisk træk, der ses ved infektiøse tarmlidelser. Det er associeret til malabsorptiv diarré på grund af mangelfuld absorption af væske og næringsstoffer fra tyndtarm. Den morfometriske undersøgelse foretaget på tarmvæv viste, at diarrégrisene havde signifikant kortere villi, dybere krypter og tyndere slimhinde i jejunum og ileum i forhold til grisene uden diarré (Manuskript I). Reduceret højde af tarmvilli medførte nedsatte villus/krypt ratioer, som antyder en negativ virkning på absorberende funktioner i tarme. Vævsprøver fra tarme fra 24 repræsentative grise blev undersøgt immunhistokemisk for celleproliferation og apoptose (Manuskript II). Resultaterne af denne undersøgelse viste at villusatrofi var associeret til forstørrelse af den proliferative del af krypterne og at epitel-turnover var forøget i diarrégrisene.

Potentielt patogene bakterier såsom *Escherichia coli*, *Enterococcus* spp., *Clostridium perfringens* og *Clostridium difficile*, er blevet mistænkt for at være involveret i NNPDS. Fluorescence *in situ* hybridisering med oligonukleotide rRNA prober specifikke for disse bakterier, blev anvendt på formalin fikseret tarmvæv indstøbt i paraffin (Manuskript III). Resultaterne af denne undersøgelse viste at adhærerende *E. coli* og *Enterococcus* spp. var involveret i NNPDS. Disse bakterier blev påvist i 37% af diarrégrisene og der var sammenhæng mellem tilstedeværelse af disse bakterier og villusatrofi og epitel læsioner i tyndtarm. Der var ingen klar sammenhæng mellem påvisning ved FISH af *C. perfringens* og *C. difficile* og diarré.

En infektionsmodel blev etableret for at give grundlag for yderligere undersøgelser af patogenesen af NNPDS (Manuskript IV). Elleve kuld nyfødte grise blev podet med homogeniserede tarme fra fire diarré case besætninger (tidligere undersøgt i projektet) og fra én rask besætning. Diarré blev reproduceret i grise fra seks kuld og disse grise udviklede patologiske forandringer, der lignede en naturlig infektion. Hverken grise podet med homogeniseret tarme fra den raske besætning eller grise podet med homogeniseret, filtreret tarme fri for bakterier, udviklede diarré. Dette indikerer at NNPDS har en bakteriel snarere end viral natur. Der var forskelle i diarréforløbet i de eksperimentelle grise med hensyn til symptomer og

histologiske læsioner, afhængigt af inokulum brugt i forsøget. Dette antyder at andre, besætningsorienterede faktorer kan påvirke diarréforløbet.

Samlet set har dette studie bidraget til viden om patogenese af NNPDS, dog er den nøjagtige ætiologi ikke blevet fastlagt. Desuden har denne undersøgelse givet en reproducerbar model af NNPDS, der kan være grundlag for fremtidige undersøgelser af dette nye syndrom hos nyfødte pøttegrise.

1. Introduction

1.1. Thesis background

Neonatal diarrhea is one of the most common problems experienced on a pig farm. Over the last decades the pig production has become more intensive resulting in an increase in number of animals per farm and changes in management practices. This intensive farming has contributed to the increase in occurrence of neonatal diarrhea which results in economic losses and excessive use of antibiotics.

In recent years, the frequency of porcine neonatal diarrhea cases has increased in several European countries. The condition commonly reported was characterized by watery to creamy, yellowish diarrhea occurring on well-maintained farms with good management practices. The onset was usually sudden with high morbidity in a farrowing batch and relatively low mortality. This diarrhea affected piglets during the first week of life, often around second-third day after birth. The antibiotic treatment had no satisfying effect, thus this diarrhea has become an important animal welfare problem and a source of frustrations for the farmers [1-3].

So far, the etiology of this diarrhea has not been explained. None of the well-known enteric pathogens such as rotavirus A, coronavirus, enterotoxigenic *E. coli* (ETEC), *C. perfringens* type C or parasites seems to be associated with the outbreaks. It has been therefore speculated that a new disease has emerged.

A French study performed in 2009 suggested that this new form of diarrhea (referred to as enzootic neonatal diarrhea) results from problems at the sow level and is linked to prolonged farrowing and low level of IgG colostrum [4, 5]. The investigation carried out in Sweden during last few years suggested an infectious nature of this diarrhea (referred to as New Neonatal Porcine Diarrhea); however, no certain causative agents have been determined [6, 7].

In Denmark, this new form of neonatal diarrhea has been reported since around 2008 [1]. First description of this condition was based on the questionnaire survey that was conducted among Danish veterinarians in 2009 [8]. In total 64 veterinarians specialized in pig production had been asked about their perception of neonatal diarrhea in pig herds. The majority of interviewed veterinarians agreed that diarrhea incidents have increased during the recent years and that there was no obvious relation between herd health statuses, sow vaccinations, management, hygiene, breeding stock, litter size and the clinical signs. The prevalence of diarrhea within the litter was estimated to be around 70-100% and the mortality was perceived to be increased with 0-5 % above normal. Since no obvious enteric pathogens typically associated with neonatal diarrhea could be detected in the affected animals and this diarrhea has come to be an increasing problem in Danish pig production, it became obvious that further investigation on the etiology and a robust control program are urgently needed.

A detailed interdisciplinary study aiming to explain the epidemiology and pathogenesis of this diarrhea, termed New Neonatal Diarrhea Syndrome (NNPDS), had been launched in 2010. The project (here: NNPDS project) enrolled three PhD students, each working with a specific area; epidemiology, pathology and intestinal microbiota. Besides the PhD projects, the study included investigations on the potential viral etiology, involvement of clostridial toxins and identification of *E. coli* isolates from the affected piglets.

The PhD project described in this thesis was related to pathology and included microscopic investigations of the intestinal tissue from piglets affected by NNPDS aiming to increase the knowledge of the syndrome with focus on the pathogenesis and etiology.

1.2. Aims and hypotheses

The overall aims of this PhD project were:

- To characterize the histopathological changes in the intestine of piglets suffering from NNPDS.

This was done by systematically performed investigation of the intestinal tissue from piglets affected by NNPDS by using light microscopy with focus on intestinal tissue morphology (Chapter 4.1) and mucosal morphometry (Manuscript I). We hypothesized, that the piglets with NNPDS develop characteristic and consistent microscopic lesions which differ from the lesions seen in classical forms of diarrhea caused by enterotoxigenic *E. coli*, *C. perfringens* type C and rotavirus A. Furthermore, we hypothesized that morphological changes in the intestinal mucosa are associated with alterations in epithelial cell turnover. This was investigated by immunohistochemical visualization of specific cell markers in the intestinal tissue (Manuscript II).

- To determine the pathogenesis and possible etiology of NNPDS.

We hypothesized that the infectious agents play significant role in NNPDS. Association between certain potentially pathogenic bacteria and pathological changes in the intestinal tissue was investigated by fluorescence in situ hybridization (Manuscript III). In order to elucidate whether NNPDS is of a bacterial or viral etiology and to provide basis for further study on the pathogenesis, an animal model of infection was established (Manuscript IV). We hypothesized that NNPDS can be reproduced in newborn piglets by per oral inoculation with intestinal tissue homogenate derived from naturally infected animals.

2. Theoretical part

2.1. Gut function and integrity

2.1.1. Intestinal barrier function

Besides digestion and absorption of the nutrients, one of the most important functions of the intestinal tract is to provide a physiological barrier between the host and the external environment. The intestinal barrier is the first line of defense protecting the intestinal mucosal surface from a harmful adhesion and invasion by microorganisms and from the intrusion of undesired antigens. It is a complex structure made of four main components: physical, chemical, immunological and microbial. Disturbed integrity of the above mentioned components affects intestinal epithelial barrier function and is considered a key factor in pathogenesis of various intestinal diseases [9-12].

- **Physical barrier**

The intestinal surface epithelium represents a potent physical barrier between the host and substances present in the intestinal lumen. The epithelial barrier is composed of a single layer of columnar epithelial cells connected by tight junctions [13]. In pig intestine, the epithelium consists of three major types of cells: columnar absorptive enterocytes, goblet cells and enteroendocrine cells. Enterocytes are the predominant type of cells and are responsible for the final digestion and absorption of nutrients, electrolytes and water. Besides participation in the digestion, the enterocytes are directly involved in immune processes; they take up and process luminal antigens resulting in antigen degradation, translocation across the epithelium or presentation to T cells. They express receptors such as Toll-like receptors and Nod-like receptors that enable microbial recognition and activation of innate immune responses including the expression of proinflammatory cytokines and chemokines. These immune active components are essential for the anti-pathogen response. Enterocytes also express major histocompatibility complexes (MHC) class I and class II molecules required for antigen processing and presentation [11, 14, 15]. The apical plasma membrane of the enterocytes forms microvilli ("brush border") that increase the overall absorptive surface of the mucosa. Goblet cells are scattered between the enterocytes. They produce and secrete mucus that lubricates epithelial surface and peptides needed for epithelial growth and repair. Enteroendocrine cells represent about 1% of epithelial cells and are specialized in exporting peptide hormones [16-18]. In addition, in the region of lymphoid aggregates, there are specific epithelial cells called microfold cells (M cells) that differ in morphology from the columnar epithelial cells. Their apical membrane is covered by short, irregular microvilli that enable microorganisms to access the cell. Material taken up by M cells is then transmitted to the associated lymphocytes and macrophages. In fact, the M cells serve as a likely portal entry to certain pathogenic bacteria and viruses [19].

Healthy intestinal epithelium is permeable for nutrients and substances important for growth and development but at the same time prevents the passage of microorganisms and harmful antigens. In this way, the intracellular homeostasis is maintained [20].

- Chemical barrier

The intestinal epithelium is covered by a mucus layer produced in goblet cells. The mucus is composed of mucins that lubricate the epithelial surface and protect against microorganisms by limiting their contact with the host cells [17, 18]. Recent studies in mice showed the evidence that the intestinal mucus is also involved in immune response and has anti-inflammatory functions [21]. Defects in mucus layer can enhance interactions between the intestinal epithelial cells and microbiota present in the lumen and susceptibility to intestinal injury. It has been shown that mucus composition and expression can be altered by intestinal microbiota and inflammatory mediators from the host [22].

- Immunological barrier

The intestinal immune system is specialized in dual tasks of immune defense against pathogens and tolerance to dietary antigens. The gut associated lymphoid tissue (GALT) consists of both isolated and aggregated (Peyer's Patches) lymphoid follicles and contains up to 70% of the body's immune cells [23]. Immunoglobulins IgA secreted from GALT are the first line of immunological defense in protecting the epithelium from the pathogenic microorganisms and bacterial toxins [24]. In addition, IgA has been shown to regulate the composition and function of gut microbiota [25]. Besides IgA, several types of specialized cells such as intraepithelial lymphocytes, macrophages, mast cells and dendritic cells presented within the lamina propria underneath the epithelium are involved in immune response in the intestine [26].

- Microbial barrier

The intestinal tract is colonized by a large and diverse community of microorganisms, dominated by bacteria, which are known to have a critical role in intestinal functionality and overall health of the host. The intestinal microbiota participates in the digestion and fermentation of carbohydrates, production of vitamins, and development of GALT as well as prevention of colonization by exogenous pathogens and indigenous potentially pathogenic microorganisms. The mechanisms by which commensal bacteria inhibit pathogens are poorly understood, however, there is evidence indicating that both direct and indirect mechanism might be involved [27-29]. Several studies have shown that commensal bacteria can directly compete with pathogens for the limited supply of the nutrients within the intestine [30, 31]. Furthermore, the commensal bacteria are able to enhance both innate and adaptive immunity to pathogens through promoting the production of immune cells and cytokines [32-34]. Studies in germ-free mice have shown that the gut microbiota is necessary for the normal development and maturation of GALT, regulation of gut specific immune cells and intestinal immune response [35-38]. Finally, gut microbiota has been shown to directly alter epithelial tight junction protein expression and modulate gut permeability [13, 39]. A complex interplay between the host immune system and the microbiota is necessary for gut homeostasis. Any disruptions in the balance in microbial community can result in pathogens colonization and can lead to pathological immune responses.

2.1.2. Postnatal development of the intestinal barrier function

- Morphological and functional changes of the intestine after birth

Following delivery, the GI tract of the newborn is challenged with adapting to changes in nutritional conditions from receiving a continuous supply of nutrients via placenta to an independent uptake of colostrum. Thus, during the early postnatal period the intestine undergoes significant morphological and functional changes reflecting in significant growth and maturation [40-43]. It has been reported, that in neonatal piglets the most dramatic changes occur within 3 days after birth. Already within the first 24 hours the intestinal weight increases by nearly 72%, villus height increases between 33% and 90% and villus diameter increases between 14% and 51%. Further acceleration in intestinal growth is observed on day 3 of postnatal life when the intestinal weight mass increases in 70-80%, the intestinal length increases by 24% and the intestinal diameter increases by 15%. Likewise the large intestine increases rapidly after birth by 33% in colonic weight in the first 24 hours and the weight is doubled by day 3 of postnatal life [43, 44]. These changes rely on three major mechanisms: the increase of local blood flow into the intestinal mucosa, accumulation of colostrum protein in the enterocytes, and changes in epithelial turnover related to increased cell proliferation [45-47]. Rapid growth of the intestine in the postnatal period is accompanied by modifications of the intestinal epithelium. The epithelial cells of the small intestine lose the ability to absorb macromolecules and the epithelial cells of the large intestine lose ability to absorb amino acids and glucose and to synthesize digestive enzymes [48]. This remodeling of the enterocytes from immature (fetal-type) enterocytes containing cytoplasmic vacuoles to mature (adult-type) enterocytes has impact on the closure of the intestinal barrier [49]. Following morphological changes, the GI tract of the neonate undergoes functional changes regarding brush border enzymes and pancreatic enzymes activity [50]. This rapid growth and functional adaptation of the intestine is stimulated by first colostrum intake. Colostrum is a rich source of various substances such as hormones and growth promoting peptides including epidermal growth factor, insulin-like growth factors, transforming growth factor, glucagon-like peptide-2 which are were proved to play an important role in the structural development of the GI tract [48, 51-53].

- Intestinal epithelial turnover and cell cycle

Cell proliferation and apoptosis are fundamental processes important for tissue development and homeostasis. In the gastrointestinal epithelium, which is one of the most rapidly dividing tissues within the body, these two processes are closely linked and provide a constant epithelial renewal essential for maintenance of the mucosal integrity and hence prevention of infections [54-56].

In the small intestine the proliferative zone is located in the intestinal crypts. Stem cells located near the crypt base are capable of producing all cell lineages of the small intestinal epithelium. The newly produced cells migrate from the intestinal crypt towards the villus-crypt junction and onto the villi. During this journey, they differentiate into their final mature form. Once the cells passed the villus-crypt junction can no longer divide. At the villus apex the cells undergo apoptosis and in that way a steady rate of epithelial turnover can be maintained. In the large intestine the stem cells are located in the proliferative zone confined to the basal two-thirds of a crypt. Newly produced cells mature at the top one-third of a crypt and are present in their mature form at the luminal epithelial surface where they then undergo apoptosis

[55]. Proliferation studies in mice and human suggest that the entire epithelial renewal takes around three to seven days [57-59].

The process of cell proliferation (called the cell cycle) is divided into four phases: G1, S (synthesis), G2 and M (mitosis). G1 is the presynthetic gap phase in which the cell prepare for cell division. After G1 the cells enter S phase in which DNA synthesis occurs and the genetic material doubles. Immediately before mitosis the cells enter a second gap phase- G2 where further preparations for cell division take place. Finally in M phase the cell divides into two daughter cells that are genetically identical with the parental cell. During the migration from the proliferative zone (crypt) to the functional zone (villus) the cells lose the ability to proliferate. These fully differentiated; non-dividing cells are in so-called resting phase (G0) [60].

At the villus apex the enterocytes undergo apoptosis, which is a counterbalance to proliferation in the crypts. Apoptosis is a process of programmed cell death and is associated with range of morphological and biochemical changes in a cell structure such as cell shrinking, plasma membrane blebbing, cell detachment, nuclear condensation, degradation of DNA, activation of caspases that cleave protein substrates and finally formation of apoptotic bodies [61]. The apoptotic cells are quickly phagocytized by mononuclear cells. Due to the rapid phagocytosis and limited release of intracellular components into the extracellular space, there is no inflammatory reaction associated with programmed cell death [62, 63].

In neonatal animals, the ratio between mitotic and apoptotic cells is the marker of epithelial turnover and intestinal maturation. It has been shown that after first colostrum intake, the proliferation of crypt stem cells is significantly increased within first two days after birth. At the same time the number of apoptotic cells decreases, resulting in rapid increase of a total number of epithelial cells, which accounts for intensive growth of the intestinal mucosa. At week 1 of age, the ratio between mitotic and apoptotic cells decreases, and this process is associated with maturation of the gut mucosa [47]. Processes of cell proliferation, differentiation and cell apoptosis in neonates are influenced by variety of substances present in the colostrum such as hormones, growth factors and regulatory peptides [46, 47, 64].

- Development of intestinal microbiota and immune barrier

At birth, the mucosal immune system of the neonate is immature. The number of lymphocytes in the intestinal tissue is low and the immunity of the neonate depends on specific and non-specific immunity acquired via colostrum. Colostrum contains large amount of immunoglobulins including IgG and IgA and immune cells such as neutrophils, macrophages, lymphocytes as well as cytokine and other nonspecific immune factors. These maternal factors transmitted to the neonate bring supplementary immunoprotection and help for the development of the immune system [65, 66].

Besides colostrum, colonization with gut microbiota is regarded not only as a basic defense mechanism against pathogens but also a critical event for the structural and functional development of the mucosal immune system in the neonate [32-34, 67-70]. Studies on germ-free animals showed that normal development of the intestinal structure and immune functions is compromised in animals deprived of microbiota. For instance, germ-free animals develop smaller Payer's patches, lower number of intraepithelial lymphocytes, thinner intestinal villi, reduced intestinal surface area and decreased epithelial turnover [71-73].

The gastrointestinal tract of fetuses is generally thought to be sterile. The first colonization of the gut by microorganisms takes place during delivery and is induced by several factors such as the delivery mode, gestation age, exposure to antibiotics, and composition of the maternal vaginal and intestinal microbiota as well as surrounding environment [68]. The neonatal gut of the newborn is colonized with variety of microbial strains, both beneficial and harmful or potentially harmful. It has been shown that in the neonatal piglet the gut microbiota community is dominated by Clostridiaceae until 0.5 day of age and then is rapidly taken over by Streptococcaceae on day 1. Lactobacillaceae predominate the community from day 5 to day 20 whereas Enterobacteriaceae are present in significant number at days 0.25 and 0.5 [74]. The colonizing bacteria communicate with the intestinal epithelium and this communication leads to immunologic reactions. This process is termed as “microbial-epithelial crosstalk” and is essential for development of the intestinal immune barrier. The intestinal epithelial cells detect bacterial antigens or bacterial products and transduce signals from luminal microorganisms to adjacent immune cells present in underlying lamina propria such as macrophages, dendritic cells and lymphocytes [15]. Since the gut microbiota is a key factor in development of immune functions, alterations of the normal bacterial colonization in early life may predispose to intestinal disorders and infections.

2.2. Intestinal epithelial barrier damage and repair

2.2.1. Intestinal epithelium injury and dysfunction

As the mucus layer is the first physical barrier protecting the intestinal mucosa against luminal microorganisms, disruption of this barrier can be crucial and initial event in pathogenesis of intestinal infections and intestinal inflammation. Changes in goblet cells response and production of the mucus have been observed in many intestinal infections with bacteria, viruses and parasites. For instance, *Salmonella typhimurium* and enteropathogenic *E. coli* (EPEC) have evolved to produce mucus-degrading enzymes which break down mucin and digest mucus barrier enabling attachment to the host epithelium [19, 75-77]. Alterations in mucin structure have also been shown to play role in the course of rotaviral infection and spontaneous colitis in animal models [78-80]. Once the pathogens have penetrated throughout the mucus layer they attach to or invade the epithelial cells.

Epithelial barrier can be breached by variety of factors such as toxic luminal substances, inflammation, interactions with microbes, oxidative stress or pharmaceuticals. Mechanisms through which the epithelial damage occurs have been widely investigated in vitro and in vivo. Two major mechanisms of intestinal epithelial injury received the most attention in research: (i) increased gut permeability due to alteration in tight junction functions and (ii) increase in epithelial cell loss. In normal condition the tight junction proteins hold the epithelial cells together and provide a potent barrier against antigens. However, during diseases that alter the tight junction proteins, the integrity of the epithelium is disrupted. This allows antigens to penetrate into the deeper parts of the intestinal mucosa and induce an inflammatory response [13]. Studies on the cultured intestinal epithelium demonstrated that mild irritants, proinflammatory cytokines, toxins and pathogens as well as adverse environmental conditions have a negative effect on the intestinal epithelial barrier increasing intestinal permeability [81]. Many bacterial pathogens have evolved

various systems to target and disrupt tight junctions. For example, it has been reported that enterohemorrhagic *E. coli* (EHEC) and EPEC modify the epithelial surface architecture and interfere with the apical junctions of epithelial cells resulting in reduced barrier functions [82, 83].

Mechanisms of mucosal injury have also been studied on various animal models of ischemia/reperfusion injury [84-86]. These studies have shown that regardless of the nature of the ischemic event, mucosal damage shows similar pattern. It is initiated by villus contraction followed by epithelial sloughing at the villus from the villus tip toward the villus base and further towards the intestinal crypt. In the large intestine, the mechanism of ischemic injury is similar as in the small intestine: the surface epithelium is lost initially followed by damage of the intestinal crypts. Even relatively mild damage to the epithelium disrupts barrier function and can lead to translocation of the bacteria and toxin from the lumen into the deeper layers of intestinal mucosa and initiate mucosal inflammatory response. Recently, the study on mice has shown that systematic introduction of lipopolysaccharide (LPS), which is an integral component of Gram-negative bacteria, causes rapid injury of the epithelium lining the villi [87]. Damage of the epithelium by LPS is reflected in excessive epithelial cell shedding due to enhanced apoptosis and results in marked villus shortening and the onset of clinical diarrhea.

Increase in epithelial cell loss has been implicated in pathogenesis of various gastrointestinal disorders including infection and immune disorders. Damaged or dead cells are immediately exfoliated from the epithelium and then rapidly replenished by new epithelial cells produced in the intestinal crypts. However, when the loss of the epithelial cells from the villus exceeds the rate of epithelial generation in the crypt, this “pathological cell shedding” is likely to have important consequences, potentially resulting in permeability defects and villus shortening (villus atrophy) [88].

2.2.2. Epithelial restitution and proliferation

Once the epithelial barrier is disrupted, epithelial repair mechanisms must rapidly restore a continuous epithelial layer in order to prevent absorption of unwanted antigens. This rapid repair of superficial damage is accomplished by a process called restitution. Restitution begins within minutes to hours after injury and includes several local events that aim on restoration of the epithelial cells integrity and normal permeability. Restitution is initiated by villus contraction which reduces the surface area requiring repair. The epithelial cells near the wound rapidly flatten, extend membrane projections, so called lamellipodia, and migrate to reform an intact epithelial barrier. Finally, restoration of tight junctions takes place [10]. However, restitution does not involve proliferation and acts only as a protection of the denuded mucosa. Complete repair of the epithelial barrier integrity requires that the epithelial cells proliferate, differentiate and migrate into the damaged area to restore the normal epithelial architecture and function [89, 90]. Proliferation begins hours or days after the injury and proceeds until the number of epithelial cells is sufficient to restore the epithelial barrier. When deeper injury occurs, remodeling of the affected layers is also needed. The mechanisms of intestinal epithelial repair are complex and involve a wide range of regulatory factors including immunoregulatory cytokines and peptides, growth factors, trefoil factors produced by goblet cells and tissue repair factors expressed within the intestinal mucosa. It has also been shown that lamina propria cells such as myofibroblasts, neutrophils and enteric nerves may play role in recovery of barrier function. Additionally, recent studies emphasize the significant role of innate immunity

and the gut microbiota in maintaining intestinal homeostasis, inflammation and tissue repair [10, 89, 91-93].

2.2.3. Epithelial cell proliferation and apoptosis in pathologic conditions

Balance of proliferation and apoptosis is important for maintenance of structural and functional properties of the intestine. Any disturbances in the epithelial cell proliferation, differentiation, migration, death and shedding are associated with various intestinal conditions, including diarrhea and inflammation. A range of enteric pathogens affect the intestinal epithelium cells which results in an excessive epithelial cell shedding. For instance, some viruses, e.g. rotavirus, or parasites such as *Strongyloides*, cause damage to the epithelial cells lining the villi [94-96]. Bacteria, such as *Salmonella*, EPEC or *Shigella dysenteriae* can cause damage of the intestinal epithelium by inducing inflammation and enhancing apoptosis of the absorptive enterocytes [97]. In large intestine, disturbances in cell division and apoptosis processes can result from infection with *Salmonella* and enteroinvasive *E. coli* [98, 99]. Similarly, bacterial toxins, for instance verotoxin from verotoxin-producing attaching and effacing *E. coli* (AEEC) and *C. difficile* toxins are capable of inducing apoptosis in the epithelial cells [100, 101]. An enhanced apoptosis of the epithelial cells affected by these microorganisms or their toxins is a response to the infection and can be a way to eliminate infected cells harboring the pathogen. Damaged or dead cells are eliminated from the epithelium which results in changes in a size and shape of the villi and enterocytes. The villi become stunted and short (atrophic). In order to compensate the epithelial cell loss, the proliferative activity of the intestinal crypts increases. This accelerated turnover rate is a major response to the epithelial cell injury and results in the rebuilding of the epithelium and recovery from villus atrophy within a few days (Figure A). However, some pathogens, for instance parvoviruses, can cause damage to the proliferative compartment in the crypts by stimulating death of the crypt cells or impair their mitotic capacity [102, 103]. As there are no new cells emerged from the crypts, the villi eventually become shorter or collapse. These two types of villus atrophy; with enlargement of the proliferative compartment or with damage to the proliferative compartment are commonly seen in domestic animals and this lead to malabsorptive diarrhea due to decrease in surface area available for absorption [88].

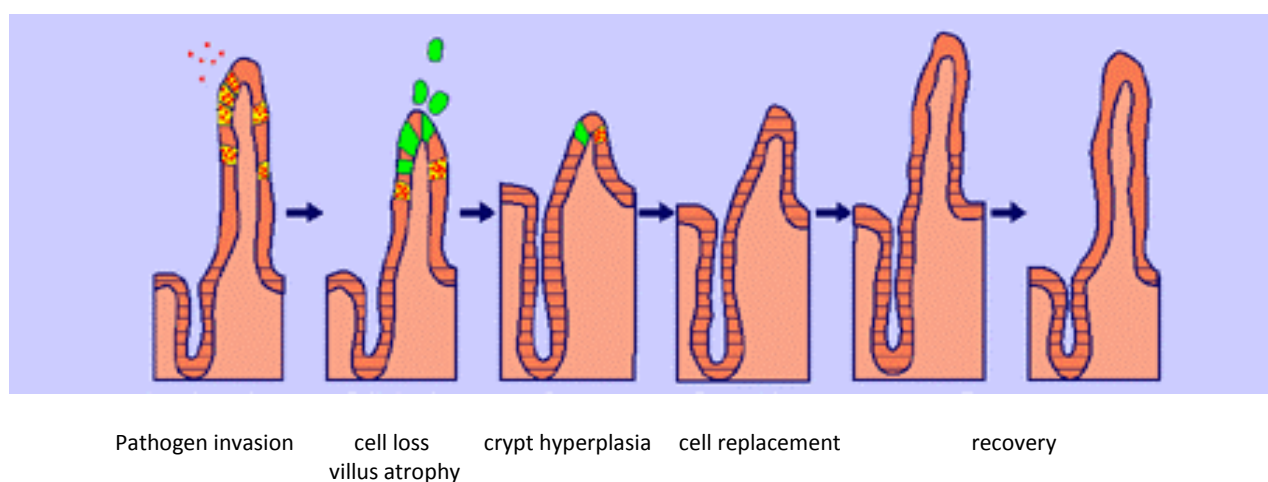


Figure A. Simplified illustration of the epithelial damage and repair in the small intestine. Figure modified from Desselberger and Gray, 2004 [94].

2.3. Neonatal porcine diarrhea

2.3.1. Basic pathophysiologic mechanisms of diarrhea

Diarrhea is one of the most common clinical signs of gastrointestinal diseases. It is defined as the presence of water in feces in relative excess in proportion to dry matter. Diarrhea in neonatal piglets is usually associated with infectious diseases and can be caused by either excessive secretion of water and electrolytes into the intestinal lumen (secretory diarrhea) or compromised intestinal epithelial barrier function [104].

Secretory diarrhea occurs when the process of secretion of ions is enhanced and exceeds absorption of fluid. This type of diarrhea is very often seen in infections with bacteria that elaborate enterotoxins. Enterotoxigenic strains of *E. coli* (ETEC) are the most common cause of this type of diarrhea in piglets. These bacteria do not invade the intestinal mucosa but adhere to the intestinal epithelium and exert a heat-labile toxin (LT) and a heat-stable toxin (ST). The overall effect of these two toxins is an increased water loss to the intestinal lumen due to blocking water absorption at the villus tips and an increase water secretion from the crypts. Water loss results in rapid acidosis and dehydration and has devastating effects on the neonate piglet [105]. Besides enterotoxins, other stimuli such as dihydroxy bile acids, hydroxylated fatty acids and inflammatory mediators can cause this type of diarrhea [106].

Diarrhea can also occur when the intestinal epithelial barrier function is compromised by loss of the epithelial cells or/and disrupted integrity of the tight junctions. For example, rotavirus, coronavirus, enteroinvasive *E. coli*, *Salmonella* and some parasites, e.g. *Cystoisospora suis*, affect the intestinal epithelial cells and cause their destruction and inflammation. Infection with these pathogens leads to excessive loss of the epithelial cells and villus atrophy, which results in malabsorptive diarrhea [94-95, 107, 108]. Due to reduction in the intestinal epithelial surface area and intestinal digestive enzyme activity, the ability of the intestine to digest and absorb material in the luminal content is diminished. The osmotic force of undigested nutrients and electrolytes results in driving water into the intestinal lumen, which leads to diarrhea. Malabsorptive diarrhea can be also aggravated by the colonic fermentation of nutrients that normally would have been absorbed in the small intestine. A sudden increase in large amounts of sugar in the colon can result in production of lactic acid. Lactic acid appears to draw water into the colon osmotically, which contributes to the severity of the symptoms during diarrheic condition [109, 110]. Diarrhea can also occur when the integrity of the tight junction is disrupted. In normal conditions the spaces between the epithelial cells are region of high sodium concentration and water moves from the lumen through the tight junctions. Simultaneously the tight junctions prevent back flow from the intracellular compartment into the intestinal lumen [111]. Some pathogens, for example *C. difficile* or EPEC can disrupt the tight junction integrity by either altering the cellular cytoskeleton or by affecting the tight junction proteins [112]. Damaged tight junctions and increased intestinal permeability result in the loss of sodium and water as well as protein into the gut lumen.

Inflammation contributes to the pathophysiology of diarrhea in most intestinal infections. For instance, prostaglandins that are produced in association with inflammation during infection with enteroinvasive

bacteria such as *Salmonella* or enteroinvasive *E. coli* have been shown to enhance intestinal secretion [107]. In fact, many of the infectious forms of diarrhea are caused by multiple mechanisms, although one of the hypersecretory, malabsorptive and inflammatory components usually predominates [112, 113].

2.3.2. Factors predisposing neonatal piglets to diarrhea

Changes in pig production over the last decades, regarding management practices and intensification of production in terms of a number of animals per farm, have contributed to the increase in occurrence of the diseases affecting the GI tract. Diarrhea can be considered the most important health and economic problem in neonatal piglets. It results in economic losses for a farmer and excessive use of antibiotics.

There are several factors predisposing newborn piglets to diarrhea. First of all, due to multifetal pregnancies, pigs are regarded as the most susceptible to intrauterine growth restriction (IUGR) among domestic animals. IUGR refers to the impaired growth and development of a fetus and its organs during pregnancy due to insufficient functionality of placenta. Previous studies showed that this condition impairs the intestinal structural and functional development and changes the bacterial colonization in the intestine of the neonate increasing susceptibility to infection [114, 115]. It has been reported that IUGR piglets had lower length and thickness of the small intestine, shorter villus length, smaller absorptive surface area, lower number of goblet cells as well as intraepithelial enterocytes compared to normally developed; normal sized piglets [116]. Undeveloped and impaired intestinal barrier function predispose to overgrowth of harmful microorganisms and, in consequence, infection. Furthermore, newborn pigs have only a small number of lymphocytes in the intestinal mucosa and the development of structures participating in immune reactions (Gut-associated lymphoid tissue) takes place subsequently after birth [65]. Additionally, the pig is born with low energy levels as it does not possess brown adipose tissue, which is a type of fat that enables newborn animals to generate a heat to maintain body temperature. In combination with the lack of a significant hair coat and very little subcutaneous tissue, the newborn piglet is susceptible to hypothermia and as a result to reduced viability or even starvation [117]. Finally, as with all of farm animals, the pig is born with generally sterile GI tract. The first colonization of the pig gut by microorganisms takes place during passage through the birth canal and is then continued throughout the suckling and nursing period [68]. The gut microbiota in the neonatal pig is therefore considered unstable and entirely influenced by the environment. Immature immune system, not well establish microbiota, low energy levels along with a low level of gastric acid and pH dependent proteases at birth, give almost free access for pathogens or potential pathogens to colonize the GI tract. Thus, in the period of first week of life, piglet performance and health entirely depend on a supply of antibodies and other immune-modulating factors which are present in colostrum. The major role of colostrum is to provide the piglet with energy and passive immunity [66]. It has been shown that the amount and quality of colostrum produced by the sow can be influenced by variety of factors such as sow breed, endocrine status, nutrition, parity, and environment. Furthermore, the amount of antibodies and immune-modulating factors available in colostrum diminishes rapidly after farrowing, especially 24 hours after birth [49, 118-120]. The intestinal absorption of the immunoglobulin from colostrum occurs mainly by non-specific endocytosis of macromolecules. However, after about 24-36 hours the intestine of the neonatal piglet is no longer able to absorb immunoglobulins due to “gut closure” [49]. Consumption of sufficient colostrum as soon as possible after birth and before gut closure is therefore crucial for piglet survival [118-121]. Failure to provide adequate passive immunity results in

relatively poorly protected pigs that are more susceptible to infection. A piglet that does not ingest an inadequate amount of colostrum will also be more sensitive to hypothermia and starvation.

Several factors can affect the colostrum intake. It has been shown that the piglets born with a higher birth weight are more competitive at the udder and can ingest more colostrum than the piglets with a lower birth weight. Furthermore, colostrum consumption was shown to be negatively correlated to the litter size [66]. Larger litters result in greater competition between piglets to reach the udder, thus the individual piglets, especially those of reduced viability, have less availability to colostrum. Finally, the environmental factors e.g. stressful management practices may also negatively influence colostrum uptake [66, 122].

2.3.3. Neonatal porcine diarrhea; overview over pathogens and pathological changes during infection

Immune compromised neonatal piglets are particularly vulnerable to infection with pathogens that are present in the environment or are normal inhabitants of the GI tract. Some of these pathogens have for decades been known to cause neonatal diarrhea and are commonly diagnosed in diarrhea outbreaks on pig farms. Other pathogens are also well known but are only occasionally being detected in neonates with diarrheic conditions. Finally, in the last years, several new pathogens have emerged. The latter ones are currently being studied and verified for their ability to cause neonatal diarrhea. However, their pathogenicity and the conditions in which they cause disease have not yet been explained.

Neonatal diarrhea is often difficult to manage due to its multifactorial nature. Variety of enteropathogens that can be involved, differences in immunity between individual animals and influence of nutritional or environmental factors often make the establishing of etiology problematic. In fact, despite improvements in diagnostic methods in past years, the exact etiology of porcine neonatal diarrhea in many cases cannot be determined [123, 124].

- **Pathogens commonly associated with neonatal porcine diarrhea**

Coronaviruses

Coronaviruses cause two clinically and histologically indistinguishable diseases in pig: Transmissible Gastroenteritis (TGE) and Porcine Epidemic Diarrhea (PED). Both diseases can affect pigs of all ages but in suckling piglets the morbidity and mortality are particularly high (up to 100%). Clinical signs of these diseases may vary depending on immunological status of the farm. Typical symptoms include acute watery diarrhea, vomiting, and dehydration. Macroscopically distended stomachs and intestines with thin and transparent wall and enlarged mesenteric lymph nodes are often seen at necropsies. The most prominent histologic lesion is villus atrophy resulting from damage of the epithelial cells infected by the virus. Other pathological changes reported from coronavirus infection include moderate infiltration with mononuclear cells and dilated capillaries, congestion and edema in the lamina propria, flattening and vacuolation of the enterocytes especially at the villus tips and luminal surface in the colon, and loss of enterocyte brush border [94, 125-128]. Neither TGE nor PED have so far been reported in Denmark [129].

Rotavirus A

Group A rotavirus is one of the most common causes of neonatal diarrhea. Severity of rotaviral infection may vary depending on dose and virulence, level of immunity in affected piglets, environmental factors and concurrent infection with other enteric pathogens. Rotavirus A typically affects piglets aged 1-7 weeks and is associated with the diarrheal syndrome known as “white scours”, “milk scours” or “3-week-scours”. In piglets less than 1 week old the infection occurs sporadically [130]. Disease resembles TGE, however morbidity and mortality is relatively low. A wide range of studies in animal models have shown that the pathomechanism of rotaviral diarrhea is multifactorial and both malabsorptive and secretory components play role [131- 134]. Malabsorptive diarrhea is due to destruction of mature absorptive enterocytes, down-regulation of the expression of absorptive enzymes, and functional changes in tight junctions between enterocytes, which leads to increased permeability. A secretory component of rotavirus diarrhea is thought to be mediated by activation of the enteric nervous system and the effects of viral enterotoxin [135-137]. Typical symptoms are white-yellow diarrhea persistent for 3-4 days, moderate dehydration, malabsorption and occasional vomiting. At necropsies a thin, transparent and flaccid intestinal wall can be observed. Microscopically villus atrophy and crypt hyperplasia especially in the jejunum and ileum are typical lesions seen in the affected animals and result from damage of the epithelial cells by the virus [95, 136, 138]. Rotaviruses are commonly present in pig population in Denmark [139].

Enterotoxigenic Escherichia coli (ETEC)

ETEC strains are recognized as the main causative agent of diarrhea in neonatal and weaned piglets. These bacteria possess two types of virulence factors essential for disease to occur: fimbrial adhesins and enterotoxins. The fimbrial adhesins target specific receptors on the intestinal brush border and enable bacteria to colonize the mucosa surface and excrete toxins. The toxins alter enterocyte functions and disturb the water and electrolyte balance in the intestine, which leads to secretory diarrhea [140-143].

Clinical signs of *E. coli* infection may appear already 2-3 hours after birth in whole litters or single piglets and vary from sudden death due to severe fluid loss to mild diarrhea without signs of dehydration. There are no pathognomonic gross or microscopic lesions associated with *E. coli* infection. Distention of the small intestine and loss of tone of the intestinal wall is sometimes observed at necropsies. The stomach is often dilated and filled with undigested milk curds. Microscopically, an adhesion of *E. coli* to the surface of enterocytes on villi can be seen, however little or no architectural change in the mucosa is associated with ETEC infection [140, 141]. However, some previous studies have reported histopathological changes such as villi shortening and epithelial lesions, mucosal swelling, cellular infiltration in the lamina propria and hyperplasia of the Payer’s patches [144, 145].

Clostridium perfringens type C

Clostridium perfringens type C causes fatal necrotizing enteritis in piglets less than 1-week-old. Beta toxin produced by this bacterium is thought to play a central role in the pathogenesis of enteritis as it is able to cause necrosis of all structural components of the villi [146, 147]. Morbidity is around 30-50% and mortality may reach 50-100%. The clinical signs vary from abdominal pain, depression and bloody diarrhea in acute and peracute conditions to non-hemorrhagic diarrhea and dehydration in chronic cases. Gross lesions in acute infection include diffuse or multifocal, extensive fibrinonecrotic enteritis especially in the jejunum,

bloody gut contents, mesenteric hyperemia and enlargement of mesenteric lymph nodes. Microscopically a varying degree of hemorrhagic necrosis of the intestinal wall with formation of pseudomembrane and the presence of large thick bacilli within the necrotic tissue is a hallmark of *Cl. perfringens* type C infection. Fibrin thrombi in blood vessels as well as diffuse edema and inflammatory exudate can also be seen throughout all intestinal layers. In chronic cases mucosal necrosis without hemorrhages is often present in the small intestines [146]. The disease seems to be eradicated on pig breeding farms by effective immunization of sows. However, the recent study performed in Switzerland showed that the pathogen can be detected in fecal samples of piglets and sows several years after an outbreak of necrotizing enteritis, despite the implemented vaccination program [148].

Cryptosporidia

Cryptosporidium spp. are intestinal protozoa associated with diarrhea in pigs aged 1 week and older and are often diagnosed together with other pathogens such as ETEC, rotavirus and coronavirus [149, 150].

Clinically cryptosporidiosis is characterized by mild diarrhea and malabsorption, however, when the disease is complicated by concomitant infections, the clinical signs may be more severe. The morbidity is usually high but mortality, if not complicated by other pathogens, is relatively low. The pathological changes of cryptosporidiosis were studied in experimentally infected piglets [151-153]. Macroscopically unspecific lesions including intestinal hyperemia and yellowish contents can be seen in the diseased animals. Microscopically villus atrophy of various severity and intestinal crypt hyperplasia is the predominant lesion. Surface epithelium is usually flattened and epithelial exfoliation from the villus tips is occasionally seen in the affected animals. Large numbers of the parasites are present on the luminal surface of the enterocytes lining the villi and occasionally intestinal crypts. The lesions are most pronounced in the distal small intestine; however they may occur in the large intestine as well. In heavily infected large intestine dilatation of the intestinal crypts, attenuation of the surface epithelium, necrotic changes and inflammatory infiltration in the lamina propria have been reported [151-153]. It is not well understood how the parasite interferes with the host cell function, but it has been suggested that the process of apoptosis may be involved in cryptosporidium infection pathogenesis [154].

Cryptosporidial infections in piglets are thought to be of little importance as the presence of these parasites is often reported from piglets with no clinical manifestation of diarrhea [150, 155, 156].

Cystoisospora suis

Cystoisospora suis (syn. *Isospora suis*) is commonly present on pig farms [157-159]. Infection occurs in piglets from 5-6 days to 2-3 weeks of age and is characterized by high morbidity and usually low, although variable mortality. This parasite is often present in mixed infections with other enteric pathogens. The clinical signs include yellowish, non-hemorrhagic diarrhea, loss of condition, dehydration and sporadically vomiting. Typical gross lesions include distention of the small intestine with yellow contents and a fibrinous or fibrinonecrotic exudate. The parasite infects and destroys enterocytes in the upper part of the villus in the small intestine and sometimes luminal enterocytes in the colon. The infected epithelial cells become degenerative and exfoliate from the villus tips leading to villus atrophy and crypt hyperplasia and necrosis. In the colon exfoliation of the infected cells on the luminal surface results in erosions [160-162].

Strongyloides ransomi

Strongyloides ransomi (pig threadworm) is a pig parasite present most commonly in warm climatic areas, especially on poor managed farms [163-165]. Infection of newborn piglets can take place by skin penetration or ingestion of the larvae shed in the colostrum of the infected lactating sow. Clinical symptoms develop after 3-7 days and include bloody diarrhea, rapid emaciation, anorexia and anemia; however severity of the symptoms may vary. Gross lesions are unspecific and include fluid intestinal contents with undigested milk. The adult worms destroy the intestinal epithelium at the base of the villi and in upper crypts and this can lead to development of villus atrophy and crypt hyperplasia. Mixed, but mainly mononuclear inflammatory infiltrate in the lamina propria and erosion in the surface epithelium are also commonly seen in the infected piglets [96, 166].

Giardia spp.

Giardia spp. infection causes malabsorptive diarrhea due to villus atrophy, shortening of microvilli, reduced digestive enzymes activity, loss of epithelial barrier function and apoptosis [167]. The infection may be manifested by pasty to fluid feces with a mucoid appearance. Gross intestinal lesions are rarely evident, although microscopic lesions such as villus atrophy, increased number of intraepithelial lymphocytes and moderate infiltration of mononuclear cells in the villus lamina propria are seen in the infected animals. Trophozoites of a crescent shape may also be present between the villi [167, 168]. *Giardia* is present in swine population, however in many cases infection of animals is asymptomatic, thus these parasites are thought to be of lesser importance [155, 169, 170].

- **Pathogens occasionally associated with neonatal porcine diarrhea**

Viral agents

Apart from group A rotaviruses, group B and C rotaviruses (so-called non-group A rotaviruses, rotavirus-like viruses, pararotaviruses or atypical rotaviruses) have been reported from cases of porcine neonatal diarrhea [171-175]. These viruses are morphologically indistinguishable but antigenically different from group A rotaviruses, therefore they cannot be detected by routine tests used for detection of group A rotaviruses. The pathogenesis of diarrhea and intestinal lesions caused by these atypical rotaviruses resemble that seen in rotavirus A infection but the clinical symptoms and pathological changes are usually less pronounced. Therefore, many naturally occurring infections with these viruses are possibly either subclinical or very mild. Atypical rotaviruses are shown to be circulating in swine populations, however their importance in the development of neonatal porcine diarrhea remains to be clarified [174, 175].

A porcine adenovirus has been occasionally reported from diarrheic piglets [176-178]. The intestinal lesions associated with adenovirus include intracellular inclusion bodies present in the epithelial cells covering usually atrophic villi over the Peyer's patches in the ileum, desquamation of the infected enterocytes and infiltration of the lamina propria with mononuclear cells [178]. The role of adenovirus in neonatal diarrhea is uncertain as the virus and inclusion bodies are also present in pigs without clinical symptoms [177].

Bacterial agents

Enterococci are part of normal intestinal microbiota, however some species e.g. *E. durans* have been reported to cause diarrhea in neonatal animals including piglets [179-184]. Some of the isolates found in pigs were later classified as new species *Enterococcus villorum* and *Enterococcus porcinus* [185, 186]. Pathogenic mechanism of diarrhea caused by enterococci is unclear as no substantial mucosal injury has been associated with *Enterococcus* infection. However, attachment of these bacteria to the luminal surface of the intestinal epithelium seems to be linked to their pathogenicity. It has been shown that under stressful growth conditions enterococci produce binding proteins that mediate adhesion to the intestinal epithelium [187]. Recently, enteroadherent enterococci were found in the Swedish study on neonatal porcine diarrhea with unknown etiology [188]. These enterococci were identified as *Enterococcus hirae* and their presence in the diarrheic piglets was associated with the histopathological changes in the intestinal mucosa such as villus atrophy, mild epithelial lesions and apoptosis of the epithelial cells [188]. Previously, *Enterococcus hirae* was reported from kittens and suckling rabbits with diarrhea [189-191].

Salmonella spp. infections are very rarely diagnosed in neonatal piglets and the importance of these bacteria in neonatal diarrhea has not been established. *Salmonella typhimurium* is the most common isolated serovar from pigs worldwide [192, 193]. Bacteria preferentially colonize the distal part of GI tract and adhere to the intestinal mucosa via fimbriae [194]. Following adhesion, *Salmonella* invades the intestinal epithelium. In experimental infections it has been shown that *Salmonella* can invade absorptive enterocytes, M-cells and goblet cells and lead to increased epithelial cell loss due to apoptosis [195, 196].

Chlamydia spp. are prevalent on pig farms, however they have been only sporadically isolated from neonatal piglets with diarrhea [197-199]. The intestinal lesions reported from both natural and experimental *Chlamydia* infection include necrotizing enteritis, villus atrophy, inflammatory cell infiltrate and lymphangitis [199-201]. Because *Chlamydia* spp. are also present in pigs without clinical manifestation of the disease, their role as enteric swine pathogens is regarded less important.

- **Emerging enteric pathogens**

Clostridium perfringens type A

In recent years, *Clostridium perfringens* type A has emerged as a potential pathogen associated with neonatal porcine diarrhea [147, 202, 203]. The pathogenesis and epidemiology of the infection is not well understood and the diagnosis is often problematic due to the fact that the bacterium is part of the normal intestinal microbiota [74]. It is believed that the pathogenicity of *C. perfringens* type A is associated with beta2 toxin produced by the bacterium, and the diagnosis of the disease is currently based on isolation of large numbers of the bacteria possessing the gene for beta2 enterotoxin and the exclusion of other known causes of neonatal diarrhea [147, 204, 205]. However, recent studies on the epidemiology of *C. perfringens* type A in a swine population have shown that the beta 2 toxin is also produced in healthy animals [206, 207]. Thus the association between this toxin and diarrhea remains to be investigated.

Infection can occur in pigs less than 1 week of age and is characterized by non-hemorrhagic diarrhea. At necropsy, thin-walled and sometimes gas-filled small intestine with mild mucosal inflammation can be seen in the diseased piglets, however, in many cases no gross lesions are observed. Typical microscopic lesions

are present mostly in the jejunum and ileum but can also spread to the colon and include: epithelial desquamation at the villus tips, local fibrin exudate, mild necrotizing enterocolitis and occasionally formation of pseudomembrane. Necrosis is often confined to the intestinal mucosa and rarely affects other layers of the intestine in contrast to the type C infections. Gram-positive bacilli are often present in the affected mucosa [147].

Clostridium difficile

Clostridium difficile has been recently recognized as an important pathogen in pig neonatal diarrhea worldwide [203, 208-211]. The bacterium produces toxins A referred to as enterotoxin and B classified as a cytotoxin, both being essential virulence factors. Toxin A is associated with extensive tissue damage and accumulation of fluid in the intestine whereas toxin B is an effective cytotoxin [212]. In gnotobiotic piglets both toxins administered systemically are able to induce severe intestinal and systematic lesions [213]. Similarly to *C. perfringens*, *C. difficile* colonizes the pig GI tract within first hours of life and almost all the piglets in some herds are colonized within 48h [214]. In humans *C. difficile* is the major cause of antibiotic-associated diarrhea and the recent study in mice demonstrated that the use of antibiotics is essential for inducing disease and histologic lesions [215-217]. However, it has been shown that in neonatal piglets antibiotic treatment does not play a role in the development of *C. difficile* infection [218].

Clinical signs of *C. difficile* infection include diarrhea and sometimes respiratory distress. At necropsy, flaccid and dilated large intestines with yellowish-brown, soft to semifluid contents, mesocolonic edema and occasionally ascites and hydrothorax can be observed. Microscopic lesions associated with *C. difficile* infections are usually seen in the large intestine and include loss of goblet cells with increased crypt mitotic activity, neutrophil infiltration of the lamina propria and multifocal erosions in the epithelium. So-called 'volcano' lesions formed by neutrophil, mucus and fibrin exudation into the colonic lumen are thought to be a hallmark pathological feature of *C. difficile* infections [208, 217-220].

Non-enterotoxigenic Escherichia coli (non-ETEC)

E. coli isolates positive for ETEC-related toxins but negative for known classical fimbrial adhesins have been previously reported from cases of pig neonatal diarrhea [221, 222]. These results raised speculations that an emerging group of diarrheagenic *E. coli* must exist in piglets. A number of studies focused on investigation of the virulence factors of *E. coli*. The results of these investigations suggested a potential pathogenic role of other non-ETEC *E. coli* e.g. enteroaggregative *E. coli* (EAEC) and attaching and effacing *E. coli* (AEEC) and indicated importance of the virulence factors such as enteroaggregative heat-stable enterotoxin 1 (EAST1) and adhesin involved in diffuse adherence (AIDA-I) [223-225].

Enteroaggregative *E. coli* (EAEC) adheres to the intestinal mucosa via fimbriae and produces toxins and inflammatory response that results in damage to the mucosa and intestinal secretion. A heat-stable enterotoxin 1 (EAST1) have been proposed as a virulence factor implicated in pathomechanism of EAEC infection [226]. In a gnotobiotic piglet model EAEC has been shown to induce diarrhea and sometimes death, moderate hyperemia of the distal part of the small intestine and cecum, and swelling of the intestinal villi [227]. Further studies have shown that EAST1 is present in pathotypes other than EAEC, such as human ETEC, enteropathogenic (EPEC) and diffusely adherent (DAEC) *E. coli* and also in ETEC strains from pigs and calves with diarrhea [226]. Recently, the studies on the prevalence of virulence factors of *E. coli* in

piglets have showed that most of *E. coli* isolates found in piglets carry gene coding for EAST1, however the association between the presence of EAST1 and diarrhea have not yet been established [228, 229]. Attaching and effacing *E. coli* (AEEC), can adhere intimately to the intestinal mucosa and efface the microvilli of the epithelial cells. These lesions have in the past been demonstrated in natural cases of diarrhea and experimental infection in neonatal and weaned piglets [230-234]. The adherence of these bacteria to the enterocytes is mediated by an outer membrane protein refer to as intimin which is also present in EPEC and some Shigatoxin-producing *E. coli* (STEC) [141]. It has been shown *that* some strains of EPEC causing attaching and effacing lesions possess adhesin involved in diffuse adherence (AIDA-I) [235]. In experimental infections in newborn piglets AIDA-I has been shown to cause diarrhea [236, 237]. It is also frequently found in *E. coli* strains that cause postweaning diarrhea and edema disease [225, 238, 239]. The histological lesions associated with EAEC include intestinal hyperemia, edema, congested blood vessels and aggregated bacteria attached to the epithelium in the distal half of the small intestine [240].

Emerging porcine coronaviruses

Recently, Swine Novel Enteric Coronavirus Disease (SECoV) outbreak has been reported across the United States. The clinical signs of the diseases include diarrhea, vomiting and a high, around 50-100% mortality of infected piglets [241]. This emerging disease is clinically indistinguishable from TGE and is caused by emerging porcine coronaviruses, including Porcine Epidemic Diarrhea virus (PEDv) and Porcine Delta Coronavirus (PDCoV). The novel PDCoV was first reported in China in 2012 [242, 243]. It was subsequently detected in the United States in early 2014, followed by detections in Canada. So far, PDCoV has not been reported in any other countries [241].

Other enteric viruses

Other viruses that can cause neonatal diarrhea in children and some animal species e.g. sapoviruses, noroviruses, enteroviruses, astroviruses and caliciviruses are found to be circulating in pig population, however their significance in enteric disease remains poorly defined [242-249].

3. Methodology overview

The work described in this includes the microscopic investigations performed on the intestinal tissue from 101 piglets collected from four Danish pig farms affected by NNPDS and the experimental infection study performed on 11 litters of piglets (n=140) aiming on reproducing clinical signs and pathological lesions of NNPDS.

Two main objectives were investigated in the PhD project. The first objective was to determine whether NNPDS is associated with the pathological changes in the intestinal mucosa and if so, whether these changes differ from the lesions seen in classical forms of diarrhea caused by well-known enteric pathogens such as ETEC, *C. perfringens* type C, rotavirus A, coronavirus. This was investigated by systematically performed histopathological, morphometrical and immunohistochemical examination of the tissue samples from the diarrheic (n=51) and non-diarrheic (n=50) piglets collected from four farms affected by NNPDS. The second objective was to identify the possible etiology of NNPDS. Firstly, we investigated whether potentially pathogenic bacteria such as *E. coli*, *Enterococcus* spp, *C. perfringens* and *C. difficile* were associated with NNPDS. This was done by the means of fluorescence in situ hybridization of the tissue samples with the specific probes detecting above mentioned bacteria. Furthermore, an animal experiment was conducted to determine whether NNPDS is of infectious nature and the diarrhea as well as the histological lesions can be reproduced in the newborn piglets challenged orally with the intestinal tissue from naturally infected animals.

Animals

The microscopic investigation was performed on the intestinal tissue samples derived from 51 diarrheic and 50 non-diarrheic piglets, aged 3-7 days (age matched within herds), collected from four Danish herds experiencing problems with neonatal diarrhea, suspected to represent NNPDS. The selection of the farms and animals was performed as a part of the epidemiological investigation (PhD; epidemiology) and is described in details in the published paper “Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhea syndrome” by Kongsted *et al.*, 2013, presented in this thesis (pp. 121-131).

In short, the selection of the diarrheic piglets was based on the presence of fluid feces for at least two subsequent days prior to selection. From each herd 11-14 diarrheic and 12-13 non-diarrheic piglets from several litters (maximum two piglets per litter) were selected. The diarrheic piglets had diarrhea for 2-5 days and were selected from the litters expressing the highest prevalence of diarrhea, whereas the non-diarrheic piglets had no diarrhea at any time and were collected from the litters with no diarrhea or very low prevalence of diarrhea. Age of the euthanized piglets varied between the herds depending on the time-point of diarrhea outbreak. None of the piglets euthanized at the age of 3-5 days received antibiotic treatment; however the piglets euthanized at 5-7 days of age were medicated according to the herd routine. Data including herd, age, diarrheic status and duration of diarrhea were collected for each piglet.

Intestinal tissue samples

Intestinal tissue samples were collected from duodenum (approximately 5 cm distal to the pylorus), mid-jejunum, ileum (approximately 5 cm proximal to ileocecal valve) and colon (spiral colon apex) of each piglet during necropsies. The samples were placed immediately after euthanasia in 10% neutral buffered formalin for minimum of 48 hours and processed according to standard laboratory procedures. In short, transverse tissue sections from each intestinal region from each piglet were embedded in paraffin wax. Two paraffin wax blocks were prepared from each animal. One block (Block 1) contained one section of the duodenum, two sections of the jejunum, two sections of the ileum and two sections of the colon. The second block (Block 2) contained one section of the duodenum, three sections of the jejunum and two sections of the ileum. Several serial sections were then cut at 3 μ m and mounted on conventional glass slides for histopathology and morphometry and on Super frost* slides (Menzel-Gläser, Germany) for immunohistochemistry and fluorescence in situ hybridization. Sections for histopathology and morphometry were then stained with hematoxylin and eosin (HE) according to standard laboratory procedures.

- **Methods used for characterization of the histopathological changes in the intestinal mucosa**

Histopathology (Chapter 4.1.)

The histopathological investigation aimed to describe any abnormalities in the intestinal tissue and was focused on determination whether or not the pathological changes are consistent in all diarrheic piglets and differ from those seen in classical forms of neonatal porcine diarrhea.

Histopathological examination was performed by the author of this thesis being blinded to the health status of the animals. For each animal, one section of the duodenum, two sections of the jejunum, two sections of the ileum and two sections of the colon (cut from the paraffin Block 1) were examined by light microscopy. The following features were investigated: intestinal villi and crypt architecture, intestinal epithelium morphology and integrity, lamina propria cellularity. Furthermore, the histopathological examination was focused on the presence of inflammation, necrotic changes and intestinal parasites such as *Cryptosporidium* spp., *Giardia* spp., *Strongyloides* spp. and *Cystoisospora suis*. Any abnormalities in normal intestinal tissue morphology were described for each intestinal region of each animal. Criteria for scoring of the lesions are further described in Chapter 4.1.

Morphometry (Manuscript I).

Morphometric analysis is widely used in research on gastrointestinal disorders since it is a quantitative method and thus more reliable and reproducible than a subjective routine histopathological assessment [250-252]. In this study the morphometric investigation was implemented in order to assess villus atrophy present in the intestinal tissue as determined by histopathology. Villus atrophy is one of the most common pathologic features associated with gut disorders in animals suffering from infectious diseases and malnutrition that occurs in weaned piglets. In those conditions villus shortening leads to reduction of the mucosal area available for absorption and, in consequence, impaired digestive and absorptive functions of

the intestine [88]. We hypothesized that the diarrheic piglets had shorter villi compared to non-diarrheic piglets and if so, the pathomechanism of NNPDS was most likely related to malabsorptive diarrhea due to reduced absorptive functions of the intestinal mucosa.

The measurements were performed on HE stained tissue sections that were previously investigated histopathologically. However, when the expected number of measurements could not be obtained due to lack of adequate vertical orientation of the villi or crypts, an additional, HE stained glass slide (cut from the paraffin Block 2) with one section of the duodenum, three sections of the jejunum, and two sections of the ileum was prepared and examined.

The morphometrical investigation was performed by the author of this thesis being blinded to the health status of the animals. The measurements were performed on the microscopic images taken by the means of an Axio Imager M1 microscope equipped with AxioCamMR digital video camera (Carl Zeiss, Oberkochen, Germany). This investigation included measurements of mucosa thickness (from the tip of the tallest villi to the muscularis mucosa using 2,5x objective), villus height (from the villus tip to the villus-crypt junction using 10x or 2,5x objective) and crypt depth (from villus-crypt junction to the base of the crypt using 10x objective) in the duodenum, jejunum and ileum and measurements of crypt depth (from the crypt mount to the crypt base using 10x objective) in the colon. Villus height/crypt depth ratios (V/C ratios) were then calculated for each small intestinal region for each piglet. Only vertically cut, well-oriented villi and crypts were measured, thus the number of measurements taken from each intestinal region as well as number of fields examined was determined by availability of well oriented sections. In general, we were able to take minimum four measurements of mucosa thickness, seven measurements of villus height and ten measurements of crypt depth from each intestinal region from each piglet. All mucosal parameters values were entered as raw data on the database and were then statistically compared between diarrheic and non-diarrheic piglets. Effect of age and duration of diarrhea on these parameters was investigated by a mixed model with randomized herd effect allowing for interaction between diarrhea status and age and between the duration of diarrhea and age. The models were reduced using likelihood ratio methods.

Immunohistochemistry (Manuscript II)

Immunohistochemistry was applied on the tissue samples from the selected piglets with the aim to assess the epithelial cell turnover in the course of NNPDS in relation to villus atrophy. The epithelial cell turnover was investigated by a direct demonstration and evaluation of specific cell markers for cell proliferation and apoptosis. Cell proliferation was investigated by immunohistochemical detection of the Ki-67 marker using Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 (Dako, Glostrup, Denmark, No. M724029-2). Ki-67 is a nuclear antigen expressed in all the phases of the cellular cycle except for G0. The intensity of Ki-67 expression and its location within the cell vary depending on the cell phase. The level of Ki-67 is low during G1 and early S-phase and progressively increases to reach maximum level in mitosis [253]. Apoptosis was demonstrated by using an antibody that recognizes cleaved caspase-3: Cleaved Caspase-3 (Asp175) Antibody #9661 (Cell Signaling Technology, distributor BioNordika Denmark A/S, Glostrup, No. 9661S). Caspase-3 is a protease implicated as a central effector protein produced as an inactive zymogen and cleaved to active subunits (17 kDa and 12 kDa). This process is associated with the initiation of apoptosis, thus cleaved caspase-3 is an important marker of cell entry into the apoptotic signaling pathway [254, 255].

The IHC procedures were performed using Envision™ G/2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red; Dako, Glostrup, Denmark) reagents. The staining for both antigens (Caspase-3 and Ki 67) was performed as single staining with Polymer/HRP (HRP conjugated Envision anti mouse/rabbit) as a secondary antibody and the color reaction was developed with 3, 3'-diaminobenzidine (DAB). The immunohistochemical procedures are described in Appendix A.

In total, the intestinal tissue samples from 24 piglets were investigated in this study. From each herd, 6 piglets were selected based on the histopathological score of villous atrophy; two diarrheic piglets with villous atrophy in at least one region of the small intestine, two diarrheic piglets with non-atrophic villi and two non-diarrheic piglets without villous atrophy. Examination of the tissue samples was performed blindly by the author of this thesis using 10x and 20x objectives. From each piglet, two sections of the jejunum, two sections of the ileum, and two sections of the colon (cut from the paraffin Block 1) were examined. The presence and localization of cells positive for proliferation and apoptosis markers in each group of piglets were described. Additionally, cell proliferation was investigated quantitatively as follows: Ki-67 positive cells and a total number of crypt cells were counted in 20 vertically well oriented (with evident base and crypt-villous junction/crypt mount), randomly selected crypts in the jejunum, ileum and colon. Proliferation labeling indexes were then calculated for each intestinal region in each piglet by dividing the total number of Ki-67 positive cells by the total number of cells counted from all scored crypts and multiplying by 100 (Ki-67 positive/ total number of cells counted x 100). Total number of cells, number of Ki-67 positive cells and labeling indexes were compared between the groups by t-test.

- **Methods used for determination of the possible etiology of NNPDS**

Fluorescence in situ hybridization (Manuscript III)

Fluorescence in situ hybridization (FISH) is a widely used technique in microbiology by which prokaryotic cells can be visualized in their natural environment [256, 257]. A direct visualization of microorganisms in the intestinal tissue sample helps to determine their association with the mucosa surface and may therefore have a potential value in elucidating their role in the disease. As we hypothesized earlier that NNPDS is of an infectious nature, we used FISH technique to investigate the role of potentially pathogenic bacteria such as *E. coli*, *Enterococcus* spp., *C. perfringens*, *C. difficile* in NNPDS. This investigation was performed by the means of specific oligonucleotide 16S rRNA or 23S rRNA- targeting probes directed against above mentioned bacteria.

The hybridization was carried as double hybridization with two probes: a general bacterial probe targeting *Domain bacteria* labeled with fluorescein isothiocyanate and one of the specific probes directed against *E. coli*, *Enterococcus* spp., *Cl. perfringens* or *Cl. difficile* labeled with the cyanine dye Cy3. The hybridization was performed on one section of the duodenum, two sections of the jejunum, two sections of the ileum and two sections of the colon (cut from the paraffin Block 1). The probes used in this study and the hybridization procedures are described in Appendix B.

All sections from each animal were examined blindly to the health status of the animals by the author of this thesis. Examination of the fluorescence signals was performed using an epifluorescence microscope under magnification 40x. Evaluation of fluorescence signals included determination of the amount of bacterial cells and their distribution in the tissue section. A positive score for particular bacteria was given when the hybridization signals were clearly specific and distinguishable as bacterial cells and could be identified with the specific probe. The amount of bacteria in each intestinal region was scored in a semi-quantitative manner and described as small, moderate or large amount. The presence, localization and amounts of bacteria were then statistically investigated with regard to determine the association with the diarrheic status, histopathological lesions and age. Epifluorescence microscopy, evaluation of the fluorescence signals and statistical analyses are further described in Manuscript III.

Animal infection model (Manuscript IV).

The main purpose of this study was to establish a reproducible animal model of NNPDS, which would provide basis for further investigations and establishment of prevention methods. We hypothesized the etiology of NNPDS to be of an infectious nature and that newborn piglets inoculated orally with the intestinal tissue homogenate derived from naturally infected animals will develop clinical symptoms and pathological changes characteristic for this syndrome.

In total, we performed 11 experiments during a period of one year. The piglets included in this study (n=140) were delivered naturally by 11 sows (Landrace x Yorkshire) purchased at 4-6 weeks before farrowing from a commercial Danish high health herd which had not experienced problems with neonatal diarrhea of unknown etiology. The inocula for challenging the piglets were prepared from the intestinal tissue from ileum and colon of the diarrheic piglets (n=51) from the field study. The tissue samples were previously collected at the necropsies, stored at -80°C and thawed before the trial. Four inocula (each inoculum corresponded to one herd) were prepared by pooling the intestinal tissue from the diarrheic piglets belonging to one herd and homogenizing with sucrose-potassium-glutamate (pH 7.0). As the exact causative agent of NNPDS has not been determined by previous investigations, we decided to prepare the inocula from the whole intestinal tissue of the field cases. Additionally, in order to determine whether NNPDS is of bacterial or viral etiology, the homogenates were deprived of bacteria by filtration through series of filters with decreasing pore size from 1.45 µm to 0.22 µm. However, only inocula derived from herd 1-3 were submitted to filtration. We were not able to prepare the filtrate from the homogenate derived from litter 4 due to insufficient tissue sample size. One litter of piglets served as a control litter and this litter was challenged with the inoculum prepared from the jejunum and colon of five healthy 4-day-old piglets collected from a commercial Danish herd that did not experience problems with neonatal diarrhea. The piglets in this litter were divided in 3 groups in order to determine the maximum concentration of inoculum tolerated by piglets. One group received 5 ml of 30% inoculum; the second group received 5 ml of 10% inoculum and the third group did not receive inoculum. None of the piglets in this litter developed diarrhea at any time of the experiment, thus 5 mL of 30% inoculum was used for challenging piglets in the other litters. The inoculation was done minimum 6 hours after the delivery, so that the piglets had possibility for colostrum uptake before the trial. In general, the piglets were inoculated at the day of birth or one day after the delivery, however two litters were challenged at day 2 post partum due to technical difficulties. The inocula were administered orally in amount of 5 ml per piglet, except for the piglets in two litters challenged with the intestinal tissue filtrate. These piglets received only 3 ml, due to insufficient

amount of inocula. In each litter seven to nine randomly selected piglets received the inoculum and the rest remained non-inoculated, however both inoculated and non-inoculated animals were kept together until the termination of the experiment. In order to determine whether NNPDS can be reproduced after one passage of the inoculum through an animal, one of the litters was challenged with the intestinal tissue derived from previously experimentally infected piglets.

The clinical examination of the animals was performed daily from day 1 to day 10 post inoculation. The presence of diarrhea was noted when the consistency of feces on the rectal swab taken during the clinical examination was fluid. Necropsies were performed successively on the selected piglets from day 2 to day 12 post inoculation. The intestinal tissue samples for histopathological investigation were collected from mid-jejunum, ileum (approximately 5 cm proximal to ileocecal valve) and colon (spiral colon apex) from each piglet, fixed in 10 % neutral buffered formalin and proceed according to standard laboratory procedures. For each animal three transverse sections of the jejunum, two transverse sections of the ileum and one-two transverse sections of the colon were examined. The histopathological investigation aimed to describe any abnormalities in the intestinal tissue with a special focus on the presence of villous atrophy and epithelial lesions. The investigation was performed by the author of this thesis blindly to the treatment group and diarrheic status of the piglets. Simultaneously, during necropsies, the intestinal contents were collected from the distal jejunum and spiral colon for the microbiological tests aiming to detect *E. coli*, *C. perfringens*, *C. difficile* and rotavirus A. For the detailed description of animals, study design and experimental procedures as well as pathological and microbiological investigations, the reader is referred to Manuscript IV.

Additionally, FISH was performed on the tissue samples from 13 piglets with experimentally induced NNPDS, showing severe clinical symptoms and severe histopathological changes in the intestinal tissue. The aim of this investigation was to identify bacteria present in the intestinal mucosa of these piglets. FISH was carried out with oligonucleotide probes targeting 16S rRNA or 23S rRNA of *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* in similar manner to the previously described FISH investigation performed on the piglets from the field study. Further details are described in Chapter 5.1.

4. Characterization of the histopathological changes in the intestinal tissue of piglets suffering from NNPDS

4.1. Preliminary pathological field investigations

In this section, the histopathological investigation of the intestinal tissue from 51 diarrheic and 50 non-diarrheic piglets collected from four Danish pig herds during outbreaks of New Neonatal Porcine Diarrhea Syndrome (NNPDS) is presented. The main objective of this investigation was to characterize the histomorphological lesions in the intestinal mucosa of the piglets suffering from NNPDS.

Materials and Methods

Animals

The piglets included in this study were collected from four Danish pig herd experiencing problems with NNPDS. In total 51 diarrheic and 50 non-diarrheic piglets, aged 3-7 days (age matched within the herd) were selected from several litters (maximum two piglets per litter). The diarrheic piglets had diarrhea for minimum two days prior to selection and were selected from the litters expressing the highest prevalence of diarrhea. The non-diarrheic piglets had no diarrhea at any time and were collected from the litters with no diarrhea or very low prevalence of diarrhea. Criteria for herds and animals inclusion and selection procedures are described by Kongsted *et al*, 2013 (pp. 121-131)

Intestinal tissue samples

Tissue samples were collected from duodenum (approximately 5 cm distal to the pylorus), mid-jejunum, ileum (approximately 5 cm proximal to ileocecal valve) and colon (spiral colon apex) of each piglet during necropsies. The samples were placed immediately after euthanasia in 10% neutral buffered formalin for minimum of 48 hours and processed according to standard laboratory procedures. In short, transverse tissue sections from each intestinal region from each piglet were embedded in paraffin wax and cut at 3 µm. The sections were then mounted on conventional glass slides and stained with hematoxylin and eosin (HE) according to standard procedures.

Histopathological evaluation

For each animal one section of the duodenum, two sections of the jejunum, two sections of the ileum and two sections of the colon were examined blindly by the author of this thesis by conventional light microscopy using 10x, 20x, and whenever necessary 40x and 63x objectives.

Histopathological examination included evaluation of the following features:

- intestinal villi architecture (height, width and shape)
- crypts of Lieberkühn architecture (depth and shape)
- intestinal epithelium morphology and integrity
- lamina propria cellularity
- presence of inflammation and necrosis
- presence of parasites (*Cryptosporidium* spp., *Giardia* spp., *Strongyloides* spp. and *Cystoisospora suis*).

The pathological changes seen in each intestinal region of each piglet were described as follows:

Shortening of the intestinal villi associated with deformation of normal villus shape (e.g. flattening, widening, blunting), increased cellularity of the lamina propria and morphological changes in the epithelium (flattened; low columnar, cuboidal or squamous enterocytes) were described as villus atrophy (Figure 1 and 2).

Elongation of the intestinal crypts (crypts of Lieberkühn) associated with the presence of several mitotic figures in the crypt epithelium was described as crypt hyperplasia (Figure 3).

Any changes in the structure of the intestinal epithelium (except for flattening associated with villus atrophy) were noted as epithelial lesions (Figure 4 and 5).

The presence of neutrophils in the lamina propria (Figure 6) was scored semi quantitatively by using scale of + (mild infiltration), ++ (moderate infiltration) and +++ (severe infiltration).

Diffuse necrotic changes in the intestinal epithelium and the lamina propria lying beneath the epithelium were described as mucosal necrosis.

Results

The main histopathological findings in 101 piglets are displayed in Table 1 and Table 2.

Herd 1 (diarrheic piglets n= 13, non-diarrheic piglets n=13)

Villus atrophy associated with intestinal crypt hyperplasia was seen in eight diarrheic and one non-diarrheic piglet. In the diarrheic piglets this lesion was present either in the jejunum (n=4) or ileum (n=2) or simultaneously in the jejunum and ileum (n=2). Villus atrophy with crypt hyperplasia in the non-diarrheic piglet and was confined to the ileum only.

One of the diarrheic piglets with villus atrophy in the jejunum had also mucosal necrosis in the ileum. In this piglet the ileal mucosa was replaced by necrotic granular material and fibrin firmly adhered to the intestinal wall (piglet no. 16; Figure 7). In one piglet with villus atrophy pronounced in the jejunum and ileum additional lesions were seen in the mucosa. These lesions included: fusion of the neighboring villi, vacuolated enterocytes on the villus tips and strikingly large amounts of bacterial cells present in the intestinal lumen, space between the villi and in an intimate contact with the intestinal epithelium (piglet no 172; Figure 8).

Local infiltration with neutrophils was seen in the lamina propria of the small intestine in 5 diarrheic and 5 non-diarrheic piglets. Epithelial lesions including flattening, desquamation and small erosions in the epithelium lining the villus tips were occasionally seen in 2 diarrheic and 2 non-diarrheic piglets. In the colon mild lesions including irregular, flattened and excessively shed enterocytes were seen in the luminal epithelium in 2 diarrheic and 2 non-diarrheic piglets. No parasites were observed in any of the piglets.

Herd 2 (diarrheic piglets n=11, non-diarrheic piglets n=12)

Villus atrophy with crypt hyperplasia in the jejunum (n=1), ileum (n=2) or simultaneously in both intestinal regions (n= 6) was the predominant histopathological finding in the diarrheic piglets. Villus atrophy with crypt hyperplasia was also seen in the ileum of one non-diarrheic piglet.

In all diarrheic piglets moderate to large amounts of bacteria were present in the intestinal lumen and the space between the villi. Additionally, in ten diarrheic piglets the bacteria were also attached to the luminal surface of the enterocytes lining the villi. The lamina propria showed congestion, slight edema and small hemorrhages in the small intestine of five diarrheic piglets (Figure 9).

Mild local infiltration with neutrophils was seen in the small intestine of six diarrheic and three non-diarrheic piglets. Epithelial lesions including flattening and desquamation were seen on the villus tips in four diarrheic piglets. Six diarrheic and one non-diarrheic piglet had mild epithelial lesions in colon. This included flattening, irregular shape and excessive shedding of the luminal enterocytes. No parasites were observed in any of the piglets.

Herd 3 (diarrheic piglets n=14, non-diarrheic piglets n=13)

Villus atrophy with crypt hyperplasia was present in ten diarrheic piglets and was confined to the jejunum (n=6) or ileum (n=3) or was seen in both intestinal regions (n=1).

Additional lesions were seen in the small intestinal tissue of three diarrheic piglets and included slight edema and small hemorrhages in the lamina propria, congestion in the blood vessels located in the submucosa (Figure 10) and mild epithelial lesions at the villus tips including flattening and desquamation of the enterocytes. Local mild neutrophil infiltration was present in the small intestines of three diarrheic and two non-diarrheic piglets. In the colon mild epithelial lesions including irregular shape, flattening and excessive shedding of the luminal enterocytes were seen in six diarrheic and two non-diarrheic piglets. No parasites were observed in any of the piglets.

Herd 4 (diarrheic piglets n=13, non-diarrheic piglets n=12)

Villus atrophy with crypt hyperplasia was seen in the small intestine of five diarrheic and four non-diarrheic piglets. In the diarrheic piglets this lesion was present either in jejunum (n=1) or ileum (n=2) or simultaneously in both regions (n=2). In the ileum villus atrophy was most pronounced in the areas over the Payer's patches (Figure 11). In the non-diarrheic piglets villus atrophy was seen either in the jejunum (n= 1) or ileum (n=3).

Diffuse necrotic changes were seen in the intestinal mucosa of two diarrheic piglets. In one of these piglets (no 79) necrosis was confined to the epithelial cells lining the villi and the lamina propria underneath the epithelium in upper 1/2-1/3 part of the villus (Figure 12). These changes were present in the duodenum and jejunum. In the other piglet (no. 139) necrosis was present in the ileum and colon. In the ileum of this piglet, the necrotic tissue was confined to the areas over the Payer's patches only whereas the villi in the other areas of the ileal mucosa were atrophic. In the colon multifocal necrotic changes were confined to the upper half of the crypts forming fibronecrotic membrane (Figure 13).

Local neutrophil infiltration was seen in three diarrheic and six non-diarrheic piglets. Mild epithelial lesions including flattening, desquamation or small erosions in the epithelium at the villus tips were seen in one diarrheic and one non-diarrheic piglet. Irregular shape, flattening and excessive shedding of the epithelial cells were also seen in the colon of two diarrheic piglets. No parasites were observed in any of the piglets.

Table 1. Diarrheic piglets; histological lesions (When considered relevant, additional information regarding microbiological and fluorescence in situ hybridization (FISH) findings are presented).

Diarrheic piglets		Histological lesions				Additional information	
Herd	Piglet no	duodenum	jejunum	ileum	colon	Microbiological findings	FISH findings
1	145	-	-	VA, N++	-		
	187	N+	-	N+	IC		
	231	-	-	-	IC	<i>C. perfr. type C</i>	
	154	-	-	-	-		
	102	-	VA	N+	IC		
	72	-	-	N+	-		
	56	N+	-	-	-		Adherent <i>Ent. spp</i>
	106	-	-	VA*	IC		
	9	-	VA*, Ep(fl, sl)	VA, EP(fl, sl)	EP(fl, shed, er)	<i>C. perfr. type C</i>	Adherent <i>E. coli</i> and <i>Ent. spp</i>
	16	-	VA	NEC	x		Adherent <i>E. coli</i> and <i>Ent. spp</i>
2	59	-	VA	-	-		Adherent <i>E. coli</i>
	85	EP(fl, sl)	VA, EP(fl, sl)	-	EP(fl, shed)		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	172	-	VA*	VA	IC		Adherent <i>E. coli</i>
	9	N+	VA, N+, LP(ed)	-	EP(shed, ir)		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	27	-	-	VA, N+	EC		Adherent <i>E. coli</i>
	3	N+	VA, LP(l, h)	VA, LP(l, h), EP(fl)	LP(h), EP(ir, shed), EC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	77	-	-	VA	EP(shed), IC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	84	-	VA*	VA	EP(fl, ir), EC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	31	-	-	N+	EC		Adherent <i>E. coli</i>
	239	N+, LP(con, ed), EP(fl)	N+, LP(con, ed, l), EP(fl)	N+	-		Adherent <i>E. coli</i> and <i>Ent. spp</i>
3	236	-	VA, LP(con), EP(fl, sl)	VA, LP(con), EP(fl, sl)	IC	Rotavirus A	Adherent <i>E. coli</i> and <i>Ent. spp</i>
	92	-	VA, LP(ed)	VA, LP(ed, con)	EP(shed), LP(h), EC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	53	LP(con, ed)	VA*, LP(con, ed)	VA, LP(con, ed)	EC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	58		VA, N+	VA, EP(fl, sl)	EP(fl, shed), EC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	3		VA, N+, LP(l, ed), EP(fl, sl)	N+	-		
	12	-	VA*	N+	IC		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	71	-	-	-	EC		Adherent <i>E. coli</i>
	115	-	VA, LP(h)	LP(ed, l), EP(fl)	EP(ir, shed)		Adherent <i>E. coli</i> and <i>Ent. spp</i>
	194, 1	-	-	VA	-		
	223	-	VA		EP (ir, fl, shed)		

124, 184,	-	-	-	-	X(124)		
152	-	-	-	VA	N+, EC, EP(fl, shed)		
72	-	-	VA	VA	EP(fl, shed)		
229	-	-	VA	-	IC		
16	-	-	-	-	EC, EP(fl, ir)		
118	-	-	VA, N+, LP(l), EP(fl, sl)	LP(l, h), EP(fl, sl)	EP(ir, fl, shed)		Adherent <i>E. coli</i>
85, 188	-	-	-	-	-		
96	-	-	-	-	IC		
190	-	-	-	-	EC		
76	-	-	VA*	-	-		
5	-	-	-	VA*, EP(fl, sl)	-		
79	NEC, N+	NEC, N+	-	-	-		Adherent <i>Ent. spp</i>
201	-	-	-	-	EP(fl, shed)		
145	-	-	-	N+	EP(ir, fl, shed)		
155	-	-	-	N+	IC		
8	-	-	-	VA	IC		
139	-	-	VA	NEC, VA	NEC	<i>C. perfr.</i> type C	
134	-	-	VA	VA	-		

4

VA: mild-moderate villous atrophy with crypt hyperplasia, VA*: severe villous atrophy with crypt hyperplasia
N: local neutrophil infiltration in the lamina propria, + mild, ++ moderate, +++severe
EP: epithelial lesions at the villous tips/luminal surface in colon (fl: flattening, sl: sloughing or desquamation, er: erosions, shed: excessive shedding, ir: irregular enterocytes)
NEC: diffuse necrosis
LP: lesions in the lamina propria (h: hemorrhages, ed: edema, con: congestion, l: lacteals dilatation)
IC- irregular crypts in colon
EC- elongated crypts in colon
-: no significant lesions
X: missing sample or autolysis
Ent. spp- Enterococcus spp.
C. perfr.- Clostridium perfringens

Table 2. Non-diarrheic piglets: histological lesions (When considered relevant, additional information regarding microbiological and fluorescence in situ hybridization (FISH) findings are presented).

Non-diarrheic piglets		Histological lesions				Additional information	
Herd	Piglet no	duodenum	jejunum	ileum	colon	Microbiological findings	FISH findings
1	238	N+	-	N+, VA, EP(fl)	IC, EP(ir, fl)		
	111, 139, 189, 96	-	-	-	-		
	131	-	-	-	IC	<i>C. diff.</i>	
	155	-	-	-	EC	<i>C. diff.</i>	
	134	-	-	-	-		Adherent <i>E. coli</i>
	93	EP(fl, sl), LP(ed)	-	N+	-		
	48	-	-	-	EC, EP(ir, fl)		
	30, 123	-	-	N+	-		
2	27	N+	N+	N+			Adherent <i>E. coli</i>
	23	-	-	-	EP(fl, shed)		
	17, 215, 105, 212, 263, 138, 134	-	-	-	-		
	149	-	-	-	EC		
	98	-	-	VA, N+	-		
	253	-	-	N++	-		
	147	-	-	N+++	-		
	45, 201, 210, 79, 166, 157, 212, 35	-	-	-	X (210,166)		
3	78	N+	N+	N+	X		Adherent <i>E. coli</i>
	156, 56	-	-	-	EP(ir, fl, shed)		
	23	-	-	-	X		
	52	-	-	N+	-		
	13, 109, 160	-	-	VA	-		
	64	N+	N+	N+	-		Adherent <i>E. coli</i>
4	52	-	-	N+++	-		
	97	N+	-	N+	-		
	37, 170	-	-	N+	-		
	206	-	-	-	-		
	217	-	-	-	-	<i>C. perfr.</i> type C	Adherent <i>E. coli</i>
	218	-	-	-	-		Adherent <i>E. coli</i>
	158	LP(l, ed)	VA, N+, EP(er), LP(l, ed, con)	N+, LP(l, ed)	-		Adherent <i>E. coli</i> and <i>Ent. spp</i>

VA: mild-moderate villous atrophy with crypt hyperplasia, VA*: severe villous atrophy with crypt hyperplasia
 N: local neutrophil infiltration in the lamina propria; + mild, ++ moderate, +++severe
 EP: epithelial lesions at the villous tips/luminal surface in colon (fl: flattening, sl: sloughing or desquamation, er: erosions, shed: excessive shedding, ir: irregular enterocytes)
 LP: lesions in the lamina propria (h: local hemorrhages, ed: edema, con: congestion, l: lacteals dilatation)
 IC- irregular crypts in colon
 EC- elongated crypts in colon
 -: no significant lesions
 X: missing sample or autolysis
Ent. spp- Enterococcus spp.
C. perfr.- Clostridium perfringens
C. diff.- Clostridium difficile

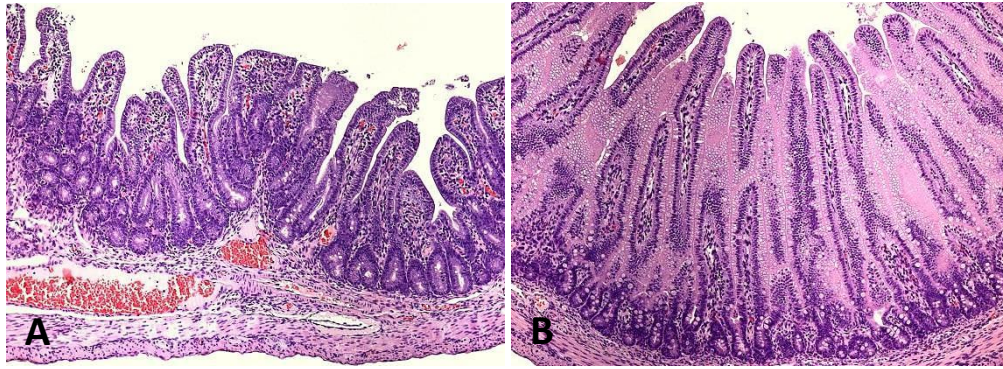


Figure 1. Intestinal mucosa from the ileum.

- A. Villus atrophy in a diarrheic piglet. Magnification 100x.
B. Normal intestinal villi in a non-diarrheic piglet. Magnification 100x.

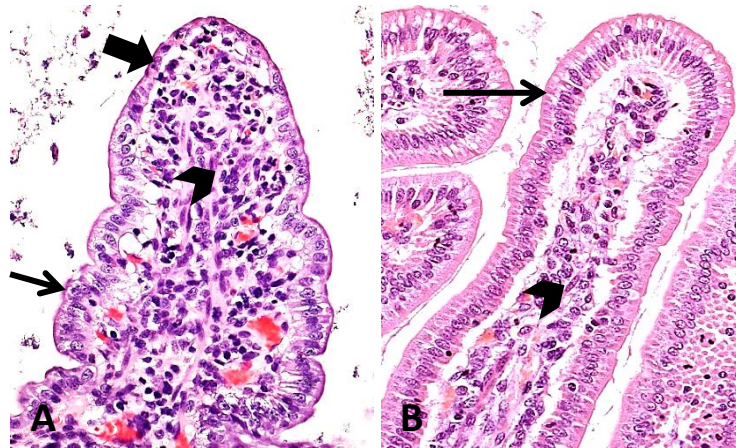


Figure 2. Intestinal villus from the jejunum.

- A. Atrophic villus in a diarrheic piglet; the villus is short, the epithelium is low columnar in the lower part (arrow) and cuboidal-squamous in the upper part (block arrow) of the villus, and the lamina propria has increased cellularity (arrow head). Magnification 400x.
B. Upper part of a normal intestinal villus in a non-diarrheic piglet. The villus is long and slender, the epithelium is tall columnar (arrow) and the lamina propria has normal cellularity (arrow head). Magnification 400x.

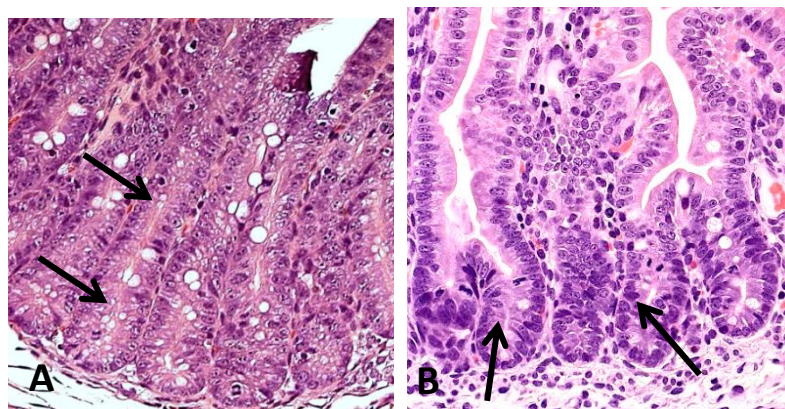


Figure 3. Crypts of Lieberkühn from the jejunum.

- A. Crypt hyperplasia in a diarrheic piglet (arrows). Magnification 200x.
B. Normal intestinal crypts in a non-diarrheic piglet (arrows). Magnification 400x.

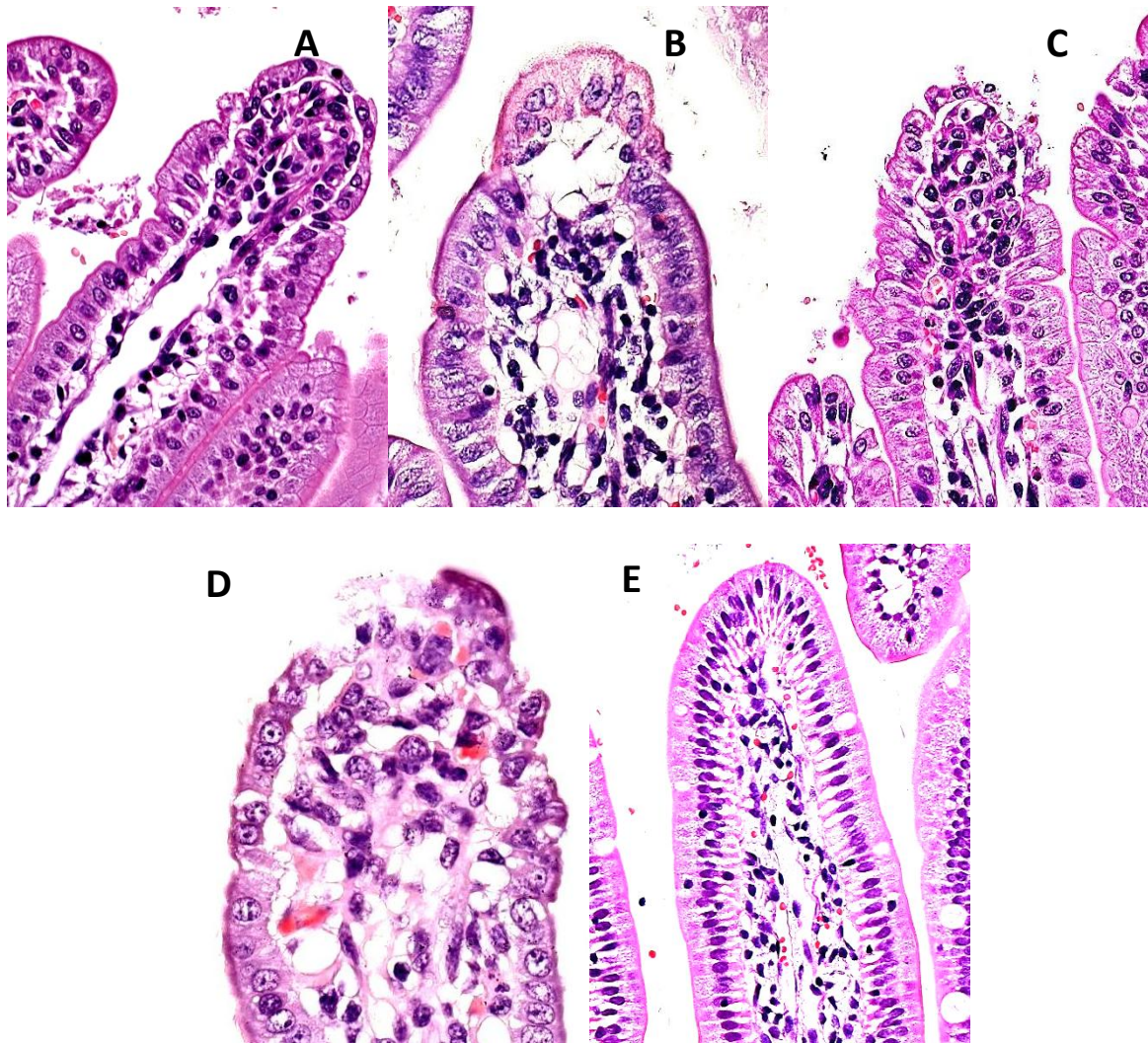


Figure 4. Representative micrographs showing epithelial lesions in the small intestinal mucosa.

- A.** Flattening of the epithelium at the villus tip. Jejunum, magnification 400x.
- B.** Desquamation of the apical enterocytes. Duodenum, magnification 400x.
- C.** Necrosis of the enterocytes at the villus tip. Duodenum, magnification 400x.
- D.** Epithelial erosions at the villus tip. Ileum, magnification 630x.
- E.** Normal small intestinal epithelium. Duodenum, magnification 400x.

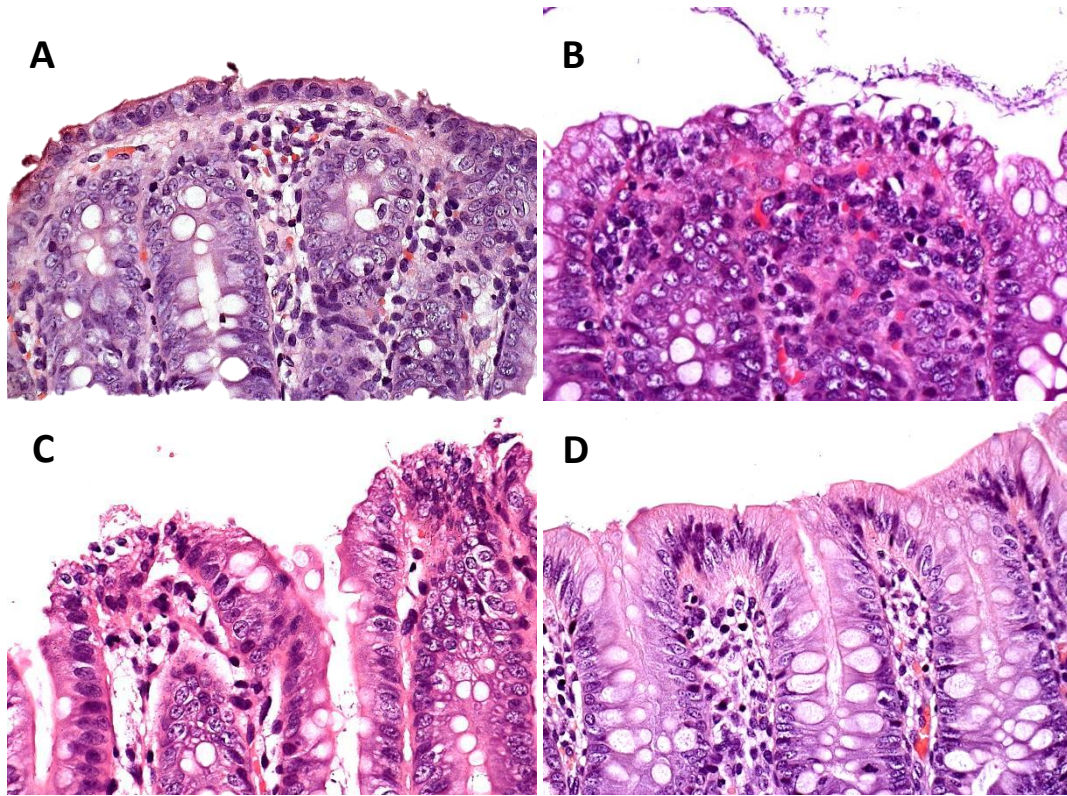


Figure 5. Representative micrographs showing epithelial lesions in colon.
A. Flattening and excessive shedding of the luminal enterocytes. Magnification 400x.
B. Irregular, disordered luminal enterocytes. Magnification 400x.
C. Necrosis of the luminal enterocytes, Magnification 400x.
D. Normal colonic epithelium. Magnification 400x.

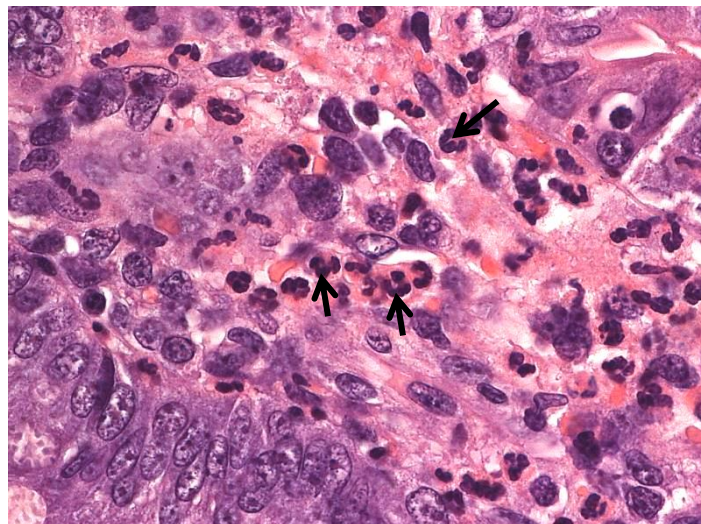


Figure 6. Neutrophil (arrows) infiltration in the lamina propria. Jejunum, magnification 630x.

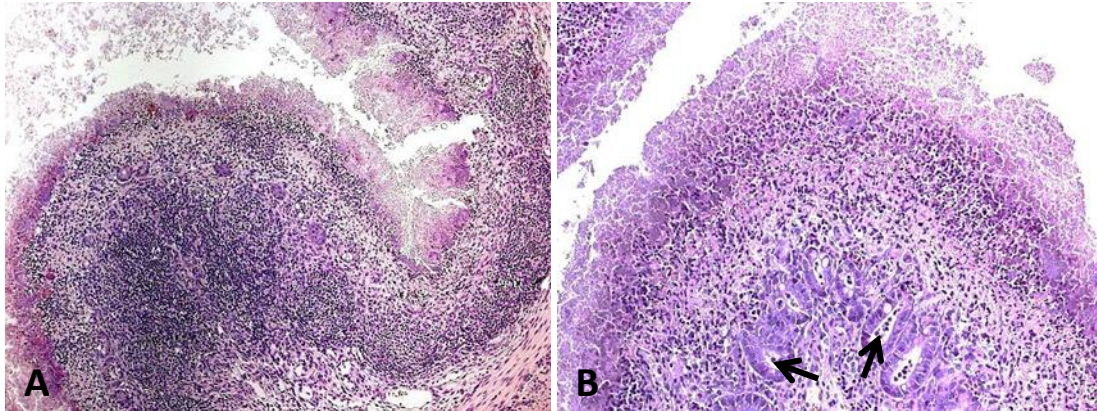


Figure 7. Necrotic ileal mucosa in piglet no 16.

A. The intestinal mucosa is replaced by necrotic tissue consisting of cellular debris, bacteria and fibrinous exudate. Magnification 100x.

B. The crypts of Lieberkühn are partially preserved (arrows). Magnification 200x

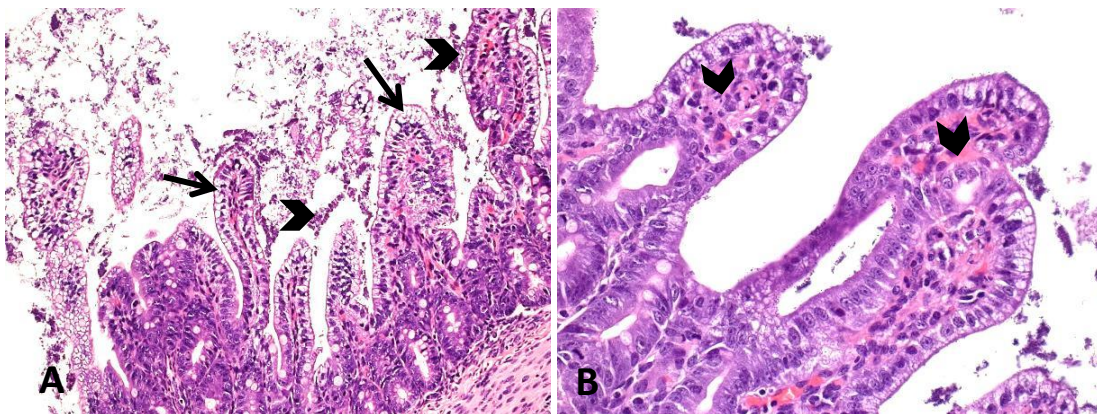


Figure 8. Jejunum of piglet no. 172.

A. The villi are atrophic and covered by vacuolated epithelium (arrows), the lamina propria is congested. Large amounts of bacteria are present in the lumen, between the villi and attach to the epithelium (arrow heads). Magnification 200x.

B. Fusion of the neighboring villi (arrow heads). Magnification 400x.

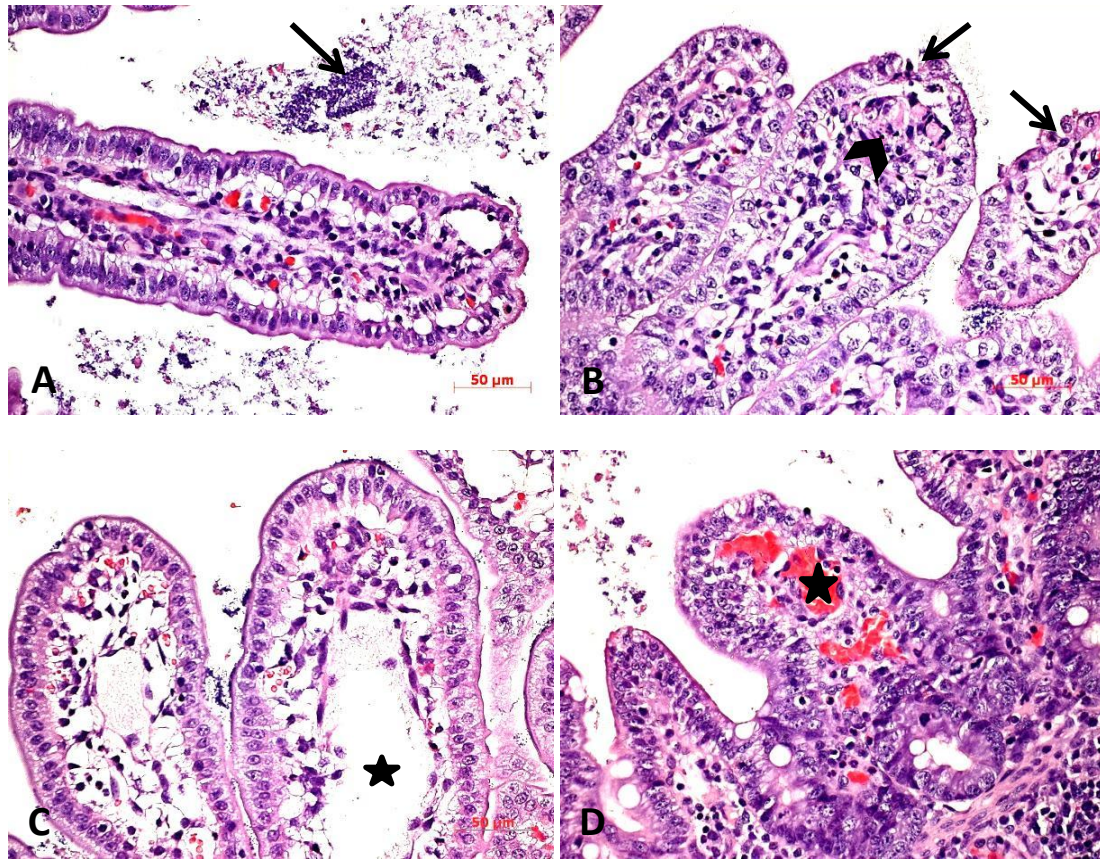


Figure 9. **A.** Intestinal villus from the jejunum of piglet no 239. Large amounts of bacteria are present in the intestinal lumen (arrow). Magnification 400x.

B. Intestinal villi from the jejunum of piglet no 236. The villi are atrophic, the lamina propria is congested (arrow head) and the epithelial lesion (flattening, desquamation and cell shedding) are present at the villus tips (arrows). Magnification 400x.

C. Intestinal villi from the jejunum of piglet no 9. The lamina propria is edematous and the central lacteal is dilated (star). Magnification 400x.

D. Intestinal villi from the jejunum of piglet no 3. Small hemorrhages are present in the lamina propria (star). Magnification 400x.

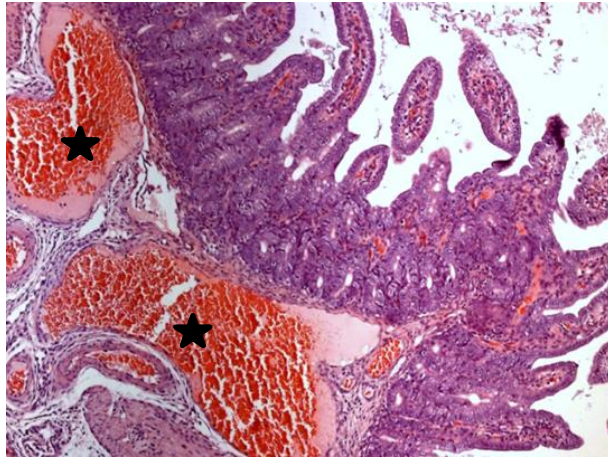


Figure 10. Blood congestion in the submucosal vessels (stars) in the jejunum of piglet no. 115.
Magnification 100x.

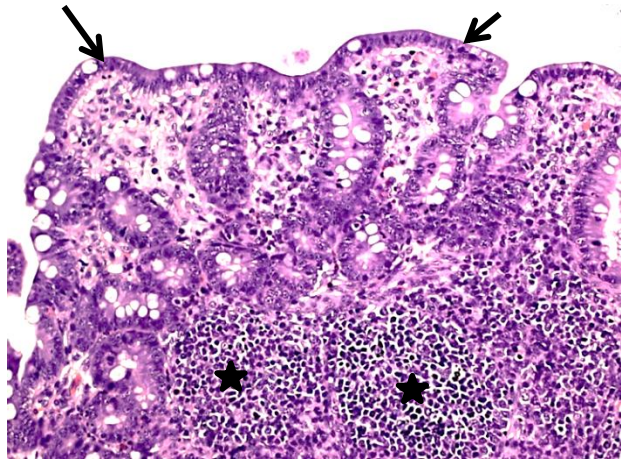


Figure 11. Severe villus atrophy (arrows) over Payer's patches (stars) in the ileum of piglet no. 190.
Magnification 200x.

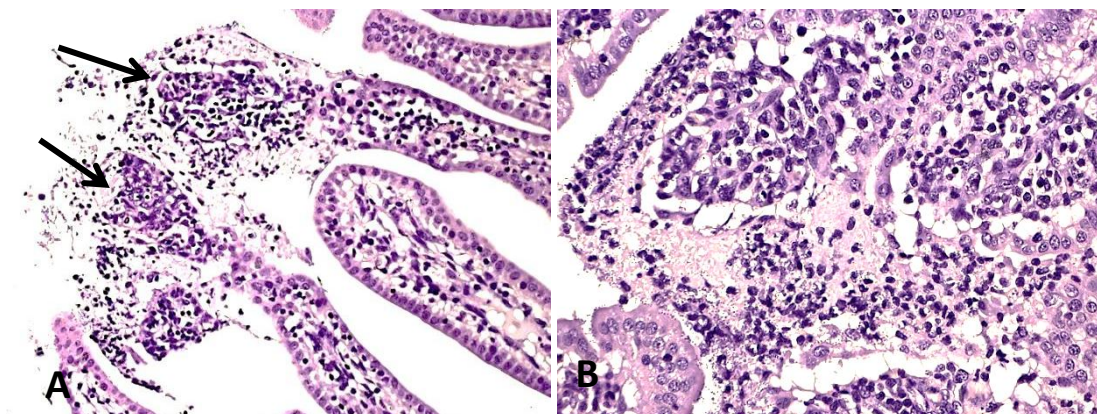


Figure 12. Necrosis (arrows) in the upper part of the intestinal villi from the jejunum of piglet no. 79.
A. Magnification 200x. B. Magnification 400x.

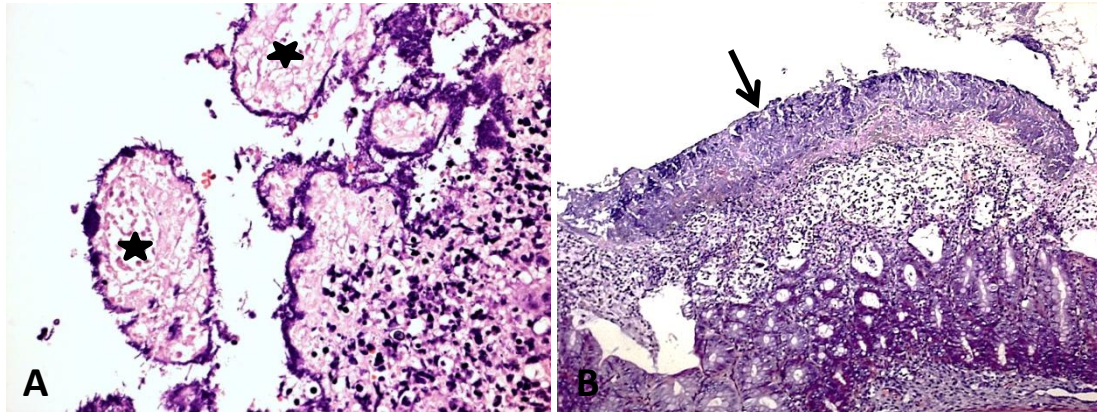


Figure 13. Necrotic mucosa in piglet no. 139.

- A.** Area over Payer's patches in the ileum. Villi remnants (stars) are surrounded by bacteria.
Magnification 400x.
- B.** Necrotic tissue forming pseudomembrane (arrow) over the intestinal crypts in the colon.
Magnification 100x.

4.1. Manuscript I

Morphometric study of the intestinal mucosa in piglets affected by new neonatal porcine diarrhea syndrome

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Morphometric study of the intestinal mucosa in piglets affected by new neonatal porcine diarrhea syndrome

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Abstract

Background

In recent years, a clinical condition characterized by non-hemorrhagic diarrhea with unexplained etiology has emerged on pig farms. This new diarrhea affects neonatal piglets less than 1-week-old, responds poorly to antibiotic therapy and occurs despite good management practices. Histopathological investigation performed on 101 piglets collected from four Danish production herds showed that the morphology of the small intestinal mucosa was altered in the piglets affected by this syndrome. These alterations were reflected in villus atrophy with concomitant crypt hyperplasia. The aim of this study was to provide morphometric characteristics of the mucosa from the neonatal piglets affected by this diarrhea. Furthermore, the aim of this study was to determine effect of age and duration of diarrhea on the mucosal parameters.

Results

The diarrheic piglets had significantly shorter villi, thinner mucosa and lower villus height/crypt depth ratios in the jejunum and ileum and deeper crypts in the entire intestine compared to age matched non-diarrheic piglets. In 69.5% of the diarrheic piglets the villi in the jejunum and/or ileum showed an atrophic pattern. Villus height and crypt depth values in these regions had tendency to return to normal with diarrhea days. The villus height values were decreased and crypt depth values were increased with age. In the colon the

crypt depth values were increased with age and in piglets aged 4, 5 and 6 days were also increased with diarrhea days.

Conclusions

The results of this study suggest that new neonatal porcine diarrhea syndrome results from decrease in mucosal area available for absorption due to shortening of the intestinal villi. The piglets affected by this syndrome develop partial, apparently regenerative villus atrophy in the jejunum and ileum and intestinal crypt deepening throughout the entire intestine. Deepening of the intestinal crypts, which are the principal site of cell proliferation in the mucosa, suggests that the epithelial turnover rate is increased in the affected piglets.

Keywords: morphometry, neonatal porcine diarrhea, villus atrophy

Background

New Neonatal Porcine Diarrhea Syndrome (NNPDS) is thought to be an emerging condition in piglets aged less than 1 week, characterized by watery to creamy, non-hemorrhagic diarrhea poorly responding to antibiotic treatment and affecting pig farms despite good management practices [1-5]. The study performed on piglets selected from four affected Danish farms, showed that this diarrhea was not caused either by rotavirus A, coronavirus, enterotoxigenic *E. coli*, *Clostridium perfringens* type C, *Clostridium difficile* or parasites (*Cryptosporidium* spp., *Giardia* spp., *Strongyloides* spp. and *Cystoisospora suis*) [5]. However, the diagnosis of the exact etiology of this syndrome has not yet been established. Histopathological investigation performed in the above mentioned study showed that the piglets suffering from NNPDS developed morphological alterations in the intestinal mucosa reflected in villus atrophy with concomitant crypt hyperplasia [5]. Villus atrophy is one of the most common pathologic features associated with gut disorders in animals suffering from infectious diseases and malnutrition that occurs in weaned piglets. In those conditions villus shortening leads to reduction of the mucosal area available for absorption and in consequence impaired digestive and absorptive functions of the intestine [6, 7]. The aim of this study was to investigate morphometric characteristics of the mucosa from the neonatal piglets affected by this diarrhea. Furthermore, the aim of this study was to determine the effect of age and duration of diarrhea on the mucosal parameters. Morphometric investigation of the gut tissue can provide data of value to elucidate the pathological mechanisms involved in this emerging syndrome with yet unknown etiology.

Methods

Animals

This study was performed on 101 piglets (51 diarrheic and 50 non-diarrheic) aged 3-7 days collected from four herds during outbreaks of NNPDS. Selection of the piglets and collection of the material is described in Kongsted *et al*, 2013 [5]. The diarrheic piglets had diarrhea for 2-5 days and the non-diarrheic piglets had no diarrhea at any time. The number of piglets arranged in groups according to age, diarrheic status and duration of diarrhea is shown in Table 1.

Tissue samples

Tissue samples were obtained from duodenum (approx. 5 cm distal to the pylorus), mid-jejunum, ileum (approx. 5 cm proximal to ileocecal valve) and colon (from spiral colon apex) during necropsies. The samples were placed in 10% neutral buffered formalin for minimum of 48 hours and processed according to standard laboratory procedures. In short, two-five serial transverse sections from each intestinal region were embedded in paraffin wax blocks, cut at 3 μ m and stained with hematoxylin and eosin (HE).

Morphometry

The intestinal tissue sections were examined by the first author blindly to the health status of the animals using an Axio Imager M1 microscope equipped with an AxioCamMR digital video camera and AxioVision Rel. 4.7.1 software (Carl Zeiss, Oberkochen, Germany). Morphometric examination of the intestinal tissue included measurements of mucosa thickness (MT), villus height (VH) and crypt depth (CD) in the duodenum, jejunum and ileum and measurements of crypt depth (CD) in the colon. Only well oriented

sections with villi and crypts cut vertically were measured. Areas with necrotic lesions were excluded from the measurement. Total MT was measured on the transverse section from the tip of the tallest villi present in the section to the muscularis mucosa. VH values were obtained by measurement of the vertically well oriented, tallest villi present in the section, from the villus tip to the villus-crypt junction. CD measurements were taken from vertically well oriented crypts from villus-crypt junction to the base of the crypt in the small intestine and from the crypt opening to the crypt base in the colon.

The number of measurements as well as the number of sections examined in each piglet was determined by availability of well-oriented sections on a glass slide. In general, for each piglet, one-two sections of the duodenum, two-five sections of the jejunum, two-four sections of the ileum and two sections of the colon were measured until the desirable number of measurements were taken. We aimed to obtain minimum four measurements of MT, seven measurements of VH and ten measurements of CP from each intestinal region in each piglet, however, in well-oriented sections we continued measurements on all available well-oriented villus-crypt units and in total a range of 4-10 measurements of MT, 7-18 measurements of VH and 10-20 measurements of CD was taken from each intestinal region in each piglet. All measurements were entered as raw data on the database and submitted to statistical analyses. Villus height/crypt depth ratios (V/C ratios) were calculated for each animal per segment by dividing overall average VH by the overall average CD.

Evaluation of villus atrophy

This was based on the histological evaluation of the villus architecture performed in the study described by Kongsted *et al.* [5] and mean VH values in the jejunum and ileum.

The tallest villi seen in the tissue specimens from the non-diarrheic piglets were considered the standard of maximum villus height. The piglets, in which the villi were tall and slender and their height values corresponded to the maximum villus heights in age matched non-diarrheic piglets, received grade 0. When the normal villus architecture was preserved but the villi were slightly shorter (approx. 60-90% of the maximum VH in age matched non-diarrheic piglets), the piglets received grade 1. Grade 2 was given to the piglets in which the villi were deformed (short and blunted) and the epithelium lining the villi was low columnar or cuboidal. VH values in this group corresponded to approx. 30-60% of the maximum VH in age matched non-diarrheic piglets. Last grade (grade 3) was given to the piglets in which villi were deformed (short and blunted), the epithelium was low columnar or cuboidal, and VH values were approx. one-third of the maximum VH or less; up to total loss of villi (Figure 1). Grade 2 and grade 3 were considered villus atrophy.

Tissue specimens in which evaluation of villus atrophy based on both morphometry and histopathology was impossible in all sections (due to e.g. autolysis, necrosis) were excluded from the analysis.

Statistical analysis

Effect of diarrhea, age and duration of diarrhea on VH, CD, MT, and V/C ratios was evaluated using a mixed model with randomized herd effect allowing for interaction between diarrhea status and age and between the duration of diarrhea and age. CD, MT and V/C ratio data were log-transformed before applying the statistical analyses. The models were reduced using likelihood ratio methods. $P < 0.05$ was taken as the level of significance.

Results

Morphometry

The mean values of villus height, crypt depth, mucosa thickness and villus heights/crypt depth ratios are presented in Table 2.

I. Non-diarrheic piglets

In the non-diarrheic piglets, the villi were taller and the crypts were shallower in the jejunum compared to the duodenum and ileum. There was no significant age effect on VH in the jejunum. In the duodenum of the piglets aged less than 6 days and in the ileum the VH values were significantly decreased with age. The CD values were significantly increased with age in the duodenum and colon. There was no significant age effect on CD in the jejunum and ileum. The intestinal mucosa was thicker in the jejunum compared to the duodenum and ileum and the MT values were significantly decreased with age in each region of the small intestine.

II. Diarrheic piglets

The villi in the jejunum and ileum were significantly shorter in the diarrheic piglets compared to the non-diarrheic piglets. The VH values were decreased with age. Additionally, VH values in the jejunum were increased with the diarrhea days. This was also observed in the ileum of the diarrheic piglets aged less than 6 days. Simultaneously, the diarrheic piglets had significantly deeper crypts in the jejunum and ileum of all piglets and in the duodenum of the piglets aged 3, 4, 5 and 7 days compared to the non-diarrheic animals. CD values were increased with age in all small intestinal segments and decreased with diarrhea days in the jejunum and ileum. In the duodenum the CD values were increased with diarrhea duration of up to 3 days and were decreased for diarrhea day 4 and 5. Colonic crypts were significantly deeper in the diarrheic piglets compared to the non-diarrheic piglets except for age of 4 days. CD values in the colon were increased with age and in piglets aged 4, 5 and 6 days were also increased with diarrhea days.

In the jejunum and ileum the V/C ratio values were significantly lower in the diarrheic piglets compared to the non-diarrheic piglets and were decreased with age and increased with diarrhea days.

The small intestinal mucosa was significantly thinner in the jejunum and ileum of the diarrheic piglets compared to the non-diarrheic piglets (except for ileum of the piglets aged 4 days) and thicker, although generally not significantly, in the duodenum. MT values were decreased with age in the jejunum and ileum and the decline was faster in the diarrheic piglets compared to the non-diarrheic piglets. In the jejunum of the piglets aged 3 and 4 days, the values for MT were increased with diarrhea days. In the duodenum and ileum the duration of diarrhea had no impact on the mucosa thickness.

Evaluation of villus atrophy

Distribution of villus atrophy in the intestinal mucosa of the piglets is presented in Figure 2.

I. Non-diarrheic piglets

In this group villus atrophy associated with deformation of the villi and flattening of the epithelium was seen in the intestine of 13% piglets (6 out of 45). Villus atrophy was confined to one intestinal region only (jejunum in one piglet and ileum in five piglets) whereas the villi in the remaining region of the small intestine were of the maximum height. Based on the VH values these piglets received grade 2. In 38% piglets (17 out of 45) the villi showed a normal architecture but their height values were slightly lower compared to the villi in some other piglets of the same age (approx. 60-90% of the maximum VH in age matched non-diarrheic piglets). These piglets received grade 1. The remaining 49% (22 out of 45) piglets had villi of the maximum height in both intestinal regions and received grade 0.

II. Diarrheic piglets

In 69.5% of the diarrheic piglets (32 out of 46) the villi showed atrophic pattern in at least one intestinal region. In this group 23 piglets received grade 2 and nine piglets received grade 3 (50% and 19.5% respectively). In 24% of the diarrheic piglets (11 out of 46) the villi had normal structure but were shorter compared to the villi of the maximum heights in age matched non-diarrheic piglets in at least one intestinal region and these piglets received grade 1. In the remaining 6.5% of the diarrheic piglets (3 out of 46) the VH values in both regions were similar to the villi of the maximum heights seen in the age matched non-diarrheic piglets and these piglets were given grade 0.

Discussion

Measurements of villus heights and crypt depths have been widely performed in the research on intestinal development and pathology in animals and humans [8-11]. In pig studies, morphometric characteristics of the intestinal mucosa have been well described in relation to the weaning process and intestinal development in the early postnatal period [6, 12-15]. This study provided morphometric data on the intestinal mucosa from piglets affected by NNPDs. The pathogenesis of this syndrome has not yet been explained thus the factors which may contribute to the intestinal mucosa alterations are unknown [5].

The results of the morphometric investigation performed on the intestinal tissue from the non-diarrheic piglets showed that the villi were higher and the crypts were shallower in the jejunum compared to the duodenum and ileum. This observation is in agreement with other studies and confirms regional differences in intestinal morphology in pigs [15-17]. The main finding of this study was a significant reduction in villus height, mucosa thickness and V/C ratios in the jejunum and ileum of the diarrheic piglets compared to their age matched non-diarrheic piglets. In the majority of the diarrheic piglets the villi showed typical atrophic pattern characterized by villus deformation and epithelial flattening [7]. Simultaneously, the intestinal crypts were significantly deeper in the intestine of the diarrheic piglets compared to the non-diarrheic piglets. Crypt hyperplasia associated with villus atrophy is a frequent manifestation in enteric diseases and is thought to occur in response to prior increase in epithelial cell loss [7]. This kind of mucosal alteration has been previously reported from piglets with neonatal diarrhea caused by rotavirus, coronavirus or parasites [18-22]. In these conditions an enhanced proliferation of the enterocytes located in the intestinal crypts reflects an effort to maintain the normal villus structure previously destroyed by the pathogen. Shortening of villi leads to decrease in mucosal absorptive area and results in impaired digestion and absorption. The results of this investigation showed that a similar pathomechanism might be involved in NNPDs. The duodenal villi were spared from villus atrophy in the

course of NNPDS and this finding corresponds to those reported from the investigations of viral diarrhea, where villus atrophy was more pronounced in the posterior part of the intestine [20, 23].

The villus height/crypt depth ratio has been reported as a useful criterion to estimate absorption capacity of the small intestinal mucosa [24, 25]. Significantly lower values of V/C ratios in jejunum and ileum of the diarrheic piglets are evidence for insufficient production of new cells in the crypts in a response to high cell loss. As the normal villus architecture could not be maintained, the overall absorption and digestion in the diarrheic piglets was probably declined.

In the majority of the piglets with villus atrophy the villi were around two-three times shorter compared to the highest villi seen in the non-diarrheic piglets and could be regarded as mild to moderate atrophy. Surprisingly these alterations were also present in the intestine of six non-diarrheic piglets. It should be emphasized that the non-diarrheic piglets in this study were collected from the same herds as the diarrheic piglets and it cannot be excluded that they were selected at the early stage of the disease [5]. However, villus atrophy in five non-diarrheic piglets was limited to the ileum only, whereas the villi in the other parts of the intestines were of normal length. It is possible that a mild villus atrophy confined to the ileum could have been insufficient to impact absorptive functions of the mucosa in these piglets and therefore did not result in diarrhea.

In 30.5% of the diarrheic piglets the villi did not show an atrophic pattern. One of the possible explanations of this finding could be that other pathomechanism was associated with diarrhea in these piglets, e.g. infection with enteric pathogens that do not produce marked changes in villus pattern. On the other hand, despite of the lack of morphological alteration in villi structure, the villus heights in 15 piglets from this group were lower than the maximum heights seen in the age matched non-diarrheic piglets. Whether this slight shortening of the villi alone had impact on absorptive capacity of the mucosa remains unknown and requires investigation by using stereological approach rather than linear methods. It has been shown that in the early postnatal period the pig intestinal mucosa undergoes dramatic morphological changes reflecting in significant growth and maturation [17, 26, 27]. Xu *et al* reported that during the first postnatal day the small intestine increased up to 33% in villus height and 24% in crypt depth [8]. Other parameters such as length, diameter, total tissue weight and mucosal tissue weight also increase dramatically after birth and it has been shown that this rapid growth of the intestine is stimulated by first colostrum intake [14, 28, 29]. In consequence, any factors that have adverse influence either on the colostrum intake level or morphology, growth and maturation of the gut tissue (e.g. environmental factors, infections) can predispose the suckling piglet to malabsorption and diarrhea. However, maturation of the intestine was not investigated in this study and it is not possible to conclude whether shortening of the villi seen in some piglets was related to the impaired intestinal growth and functional development. Alternatively, these piglets could have been selected at the time of recovery when the intestinal mucosa was returning to its normal morphology.

In this study, the effect of diarrhea duration has been statistically investigated. The results showed that the villus length and V/C ratio values were increased and the crypt depth values were decreased with diarrhea days. This improvement of villus heights and crypt depths towards normal values suggests that due to regenerative capacity of the intestinal crypts villus atrophy could be reversible and normal mucosal architecture could probably be rebuilt within a few days [7]. If this is true, the functional recovery of the mucosa and in consequence clinical recovery would be expected. However, these results should be

interpreted carefully, since the number of piglets having diarrhea for longer period (4 and 5 days) was relatively low.

Morphometry performed on the tissue specimens from the colon showed that in most of the diarrheic piglets the colonic crypts were significantly deeper compared to the non-diarrheic piglets. Increased CD values might have been correlated with expansion of the proliferative compartment. If this hypothesis is true, an accelerated rate of superficial epithelial cell loss would be the most likely cause of crypt deepening and could have resulted in an impaired absorption of fluid from the intestinal lumen. Further studies are needed in order to explain crypt deepening in the piglets suffering from NNPDS.

Conclusion

The results of the morphometric investigation showed that the piglets suffering from NNPDS developed structural changes in the intestinal mucosa that included partial, apparently regenerative villus atrophy in the jejunum and ileum and intestinal crypt deepening throughout the entire intestine. Reduction in villus height frequently observed in the diarrheic piglets leads to decrease in V/C ratios and mucosal thickness which indicates an adverse effect on gut absorptive functions. Additionally, deepening of the intestinal crypts, which are the principal site of cell proliferation in the mucosa, suggest that in the course of NNPDS the epithelial turnover rate is increased.

Competing interests

The authors of this research paper have no competing interests.

Authors' contributions

TKJ and BJ participated in the conception and design of this study, and contributed to the interpretation of the results. Statistical analyses were performed by AS. All authors participated in drafting, proofreading and approval of the manuscript.

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Tables

Table 1. Number of piglets included in this study.

Age	Total no. of non-diarrheic piglets	No. of diarrheic piglets				
		Total	Diarrhea duration			
			2 days	3 days	4 days	5 days
3 days	14	15	11	4		
4 days	9	10	6	3	1	
5 days	17	17	6	6	5	
6 days	6	6		4	1	1
7 days	4	3		1		2

Table 2. Morphometric parameters (in μm) of the intestinal mucosa in neonatal piglets (mean \pm SD).

Age	3 days		4 days		5 days		6 days		7 days	
	DP	NP	DP	NP	DP	NP	DP	NP	DP	NP
duodenum	VH	723 \pm 124	698 \pm 158	755 \pm 163	755 \pm 190	683 \pm 155	654 \pm 100	610 \pm 114	698 \pm 91	802 \pm 134
	CD	159 \pm 38	147 \pm 21	193 \pm 33	167 \pm 23	182 \pm 49	203 \pm 38	197 \pm 22	218 \pm 40	187 \pm 32
	MT	945 \pm 160	874 \pm 114	960 \pm 191	885 \pm 194	845 \pm 185	817 \pm 119	818 \pm 137	919 \pm 73	875 \pm 134
	V/C	4.6 \pm 1.1	4.8 \pm 1.5	3.9 \pm 1.0	4.5 \pm 1.2	3.9 \pm 1.6	3.3 \pm 0.8	3.0 \pm 0.6	3.4 \pm 0.8	4.4 \pm 0.9
jejunum	VH	837 \pm 277	986 \pm 216	691 \pm 347	1042 \pm 216	604 \pm 263	598 \pm 194	840 \pm 165	414 \pm 85	1136 \pm 155
	CD	137 \pm 27	125 \pm 21	170 \pm 32	133 \pm 16	153 \pm 28	182 \pm 66	134 \pm 21	185 \pm 24	119 \pm 17
	MT	1057 \pm 234	1191 \pm 214	839 \pm 335	1219 \pm 226	712 \pm 238	766 \pm 203	952 \pm 138	590 \pm 105	1295 \pm 230
	V/C	6.5 \pm 2.3	8.1 \pm 2.4	4.5 \pm 2.7	8.1 \pm 1.9	4.2 \pm 2.4	3.5 \pm 1.7	6.2 \pm 1.7	2.3 \pm 0.6	9.8 \pm 2.1
ileum	VH	591 \pm 209	762 \pm 249	554 \pm 164	688 \pm 136	491 \pm 212	421 \pm 112	634 \pm 120	390 \pm 70	824 \pm 150
	CD	149 \pm 21	138 \pm 35	170 \pm 21	139 \pm 19	146 \pm 27	159 \pm 23	134 \pm 21	150 \pm 27	125 \pm 16
	MT	813 \pm 221	964 \pm 263	777 \pm 205	791 \pm 157	613 \pm 291	541 \pm 129	687 \pm 134	545 \pm 73	860 \pm 291
	V/C	4.0 \pm 1.6	5.8 \pm 2.2	3.3 \pm 1.1	4.9 \pm 1.3	3.6 \pm 1.9	2.7 \pm 0.7	4.7 \pm 1.0	2.7 \pm 0.8	6.6 \pm 1.6
colon	CD	272 \pm 59	237 \pm 48	319 \pm 54	322 \pm 57	313 \pm 61	365 \pm 70	314 \pm 36	410 \pm 70	315 \pm 55

Significant differences between diarrheic (DP) and non-diarrheic piglets (NP) are presented in bold ($p < 0.05$). VH- villous height, CD- crypt depth, MT- mucosa thickness, V/C- villous height/crypt depth ratio.

Figures

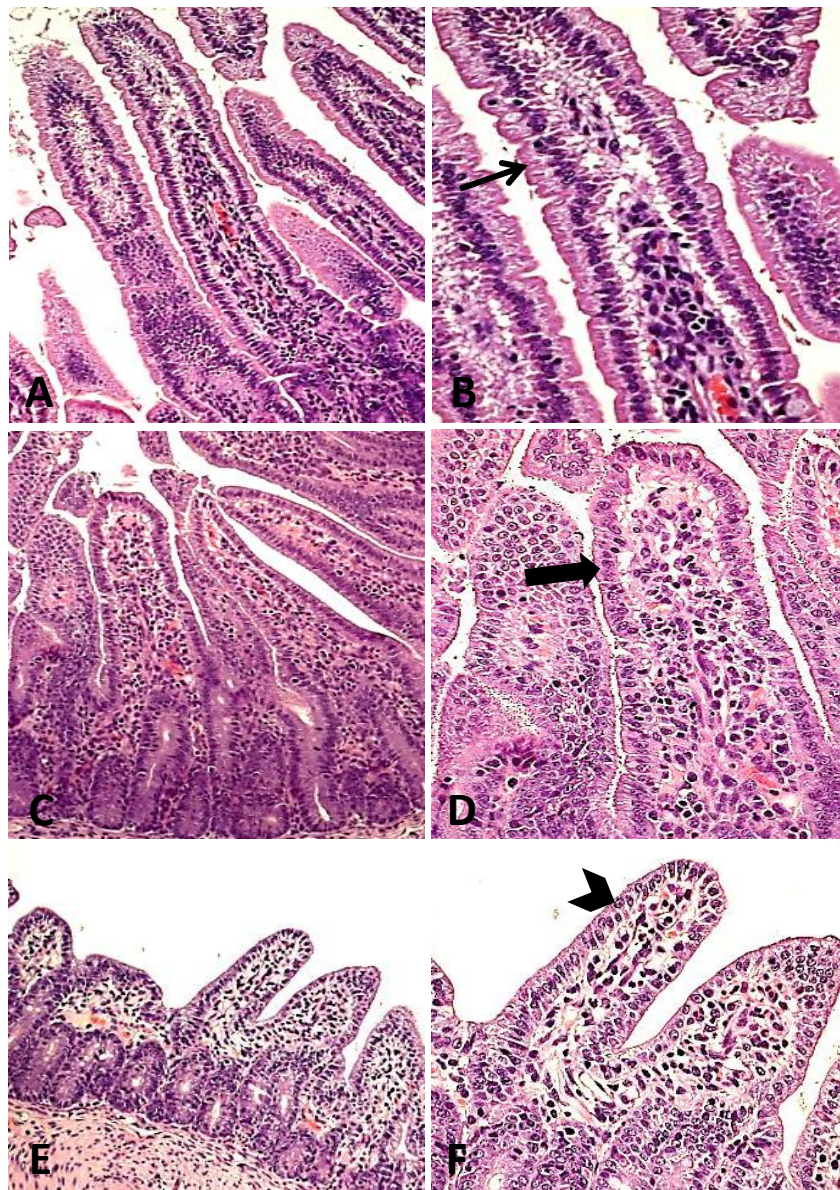


Figure 1. Representative light micrographs of HE stained sections from the jejunum showing villi alterations seen in the piglets.

A, B. Villi in the piglets that received grade 0 or 1. The villi are long and slender and the epithelium is high columnar (thin arrow). A. Magnification 100x, B. Magnification 200x.

C, D. Villi in the piglets that received grade 2. The villi are short and blunted and the epithelium is low columnar- cuboidal (thick arrow). C. Magnification 100x, D. Magnification 200x.

E, F. Villi in the piglets that received grade 3. The villi are very short or absent and the epithelium is low columnar- cuboidal (arrow head). E. Magnification 100x, F. Magnification 200x

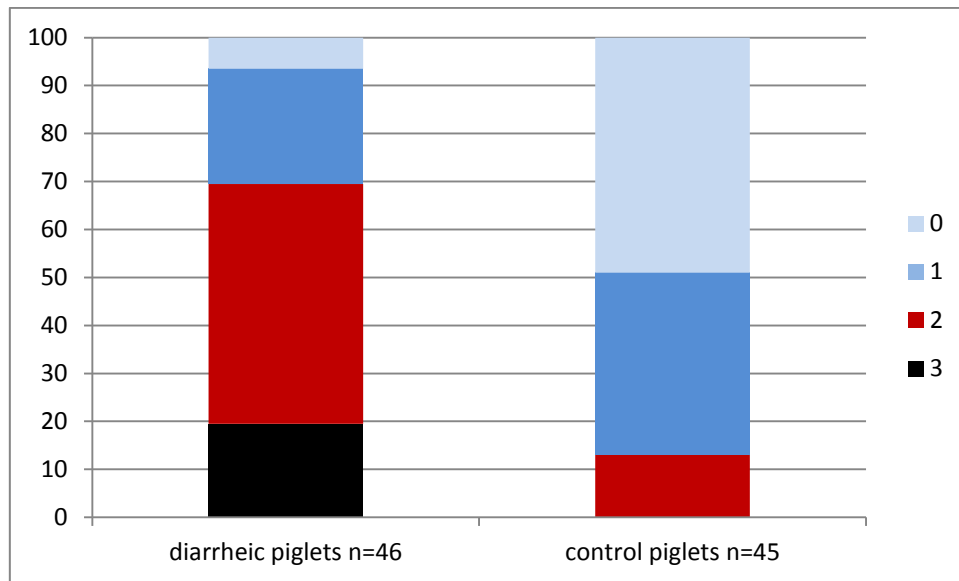


Figure 2. Prevalence (in %) of the villi alterations graded from 0 to 3, based on histopathology and morphometry.

4.2. Manuscript II

Immunohistochemical study of cell proliferation and apoptosis in newborn piglets from herds affected with new neonatal porcine diarrhea syndrome

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Immunohistochemical study of cell proliferation and apoptosis in newborn piglets from herds affected with new neonatal porcine diarrhea syndrome

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Abstract

Balance of cell proliferation and apoptosis in the gastrointestinal epithelium is important for maintenance of structural and functional properties of the intestine. A range of enteric pathogens are known to affect intestinal epithelium turnover in pig gastrointestinal tract. This study aimed to assess the epithelial cell turnover in neonatal piglets suffering from New Neonatal Porcine Diarrhea Syndrome (NNPDS) in relation to previously described alteration in mucosal architecture including villus atrophy with concomitant crypt elongation. Evaluation of cell proliferation and apoptosis was performed on tissue samples from selected piglets collected from four herds affected with NNPDS by immunohistochemical detection of Ki-67 (cell proliferation) and caspase-3 (apoptosis) markers. From each herd 6 piglets were selected based on histopathological score of villus atrophy: two diarrheic piglets with villus atrophy in at least one section of the small intestine, two diarrheic piglets with non-atrophic villi and two non-diarrheic piglets without villus atrophy. Ki-67 and caspase-3 staining pattern was as expected. The diarrheic piglets with villus atrophy had significantly higher number of crypt cells in the jejunum and cells positive for Ki-67 in the jejunum and ileum compared to the non-diarrheic piglets. Additionally, the number of Ki-67 positive cells was significantly higher in the jejunum of the diarrheic piglets with villus atrophy compared to the diarrheic piglets with normal villi. This indicates that villus atrophy developed in the diarrheic piglets suffering from NNPDS is a type of a crypt hyperplastic villus atrophy associated with enlargement of the proliferative compartment. Additionally, in half of the diarrheic piglets with villus atrophy the occurrence of apoptotic cells was more frequent compared to the piglets with non-atrophic villi. These findings suggest that epithelial turnover rate was increased in the course of NNPDS.

Keywords: apoptosis, caspase-3, cell proliferation, Ki-67, immunohistochemistry, neonatal porcine diarrhea, villus atrophy

Introduction

The intestinal epithelium is exposed to both dietary and environmental antigens, and for many pathogens the epithelial cells are a route of entry into the host. Therefore, the intestinal epithelium has multiple innate defense mechanisms that protect the intestine from the invasion of the microorganisms. The integrity of the epithelium and the constant renewal of epithelial cells are critical defense mechanisms. The intestinal mucosa exists in a highly dynamic state. Epithelial cells are generated from stem cells at the bottom of crypts and then migrate upward to the tip of the villi, undergoing proliferation, differentiation, maturation and apoptosis [1-3]. In addition to this basal physiological renewal, the epithelium can also turnover in response to various stimuli, including immune disorders, gut microbiota, and pathogens [4, 5]. In pigs, a range of enteric pathogens are known to affect intestinal epithelium turnover and cause a disease. For instance, infection with rotavirus, coronavirus, *Salmonella*, attaching and effacing *E. coli* or cryptosporidia may lead to the increase in loss of the epithelial cells due to a direct damage of the epithelium or enhanced apoptosis of the enterocytes [6-10]. Some other pathogens e.g. parvoviruses, can cause damage to the proliferative compartment by stimulating death of the crypt cells or impair their mitotic capacity. An enhanced apoptosis of the epithelial cells affected by the microorganisms can also be a response to the infection and a way to eliminate infected cells harboring the pathogen [11-13]. Changes to the epithelial turnover result in alterations in the mucosal morphology and very often lead associated with villous atrophy [11].

New Neonatal Porcine Diarrhea Syndrome (NNPDS) is a diarrheic condition affecting piglets in the first week of life and occurring on well-managed, high health pig farms. The pathogenesis of this diarrhea is not well-understood and so far, the etiology of this syndrome has not been established [14]. The histopathological investigation performed on the tissue samples from 51 diarrheic and 51 non-diarrheic piglets collected from four Danish farms experiencing problems with NNPDS has shown that the majority of the diarrheic piglets developed villus atrophy with concomitant crypt elongation. However, none of the well-known pathogens causing villus atrophy in neonatal piglets, such as coronavirus, rotavirus or parasites were associated with this diarrhea [14]. The aim of this study was to assess the epithelial turnover in neonatal piglets from herds affected by NNPDS in relation to the previously described morphological alterations in the intestinal mucosa. Furthermore, this investigation was performed in an attempt to identify the mechanism governing intestinal villus atrophy developed in the piglets suffering from NNPDS.

Materials and methods

Tissue samples

Evaluation of cell proliferation and apoptosis was performed on tissue samples from 24 piglets collected from four herds affected with NNPDS. From each herd 6 piglets were selected based on histopathological score of villus atrophy described in Kongsted *et al.* 2013; two diarrheic piglets with villus atrophy in at least one section of the small intestine, two diarrheic piglets with non-atrophic villi (here: normal villi) and two non-diarrheic piglets without villus atrophy [14]. Evaluation of cell proliferation and apoptosis was performed on formalin-fixed, paraffin-embedded tissue specimens from jejunum, ileum and colon of each piglet.

Proliferation and apoptosis markers

Cell proliferation was investigated by immunohistochemical detection of the Ki-67 marker. Ki-67 (also known as MKI67) is a nuclear antigen expressed in all the phases of the cellular cycle except for G₀. The intensity of Ki-67 expression and its location within the cell vary depending on the cell phase. The level of Ki-67 is low during G₁ and early S-phase and progressively increases to reach maximum level in mitosis [15, 16].

Apoptosis was demonstrated by immunohistochemistry using an antibody that recognizes cleaved caspase-3. Caspase-3 is a protease implicated as a central effector protein produced as an inactive zymogen and cleaved to active subunits (17 kDa and 12 kDa). This process is associated with the initiation of apoptosis, thus cleaved caspase-3 is an important marker of cell entry into the apoptotic signaling pathway [17].

Primary Antibodies

In this study proliferation and apoptosis markers were detected by use of the following antibodies:

1. Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 (Dako, Glostrup, Denmark, No. M724029-2) was used for detection of proliferative cells.
2. Cleaved Caspase-3 (Asp175) Antibody #9661 (Cell Signaling Technology, distributor BioNordika Denmark A/S, Glostrup, No. 9661S) was used for detection of apoptotic cells.

IHC procedures

Tissue specimens were cut in 3 µm thick sections and mounted on Super frost* slides (Menzel-Gläser, Germany). Prior to IHC, the slides were deparaffinized in xylene (2x 2 min) and rehydrated in ethanol (2x2 min) and water.

Heat-induced antigen retrieval (HIER) was done in TRIS/EDTA buffer (10 mM Tris-Sigma, 1 mM EDTA Titriplex III, pH 9,0- Merck, Darmstadt, Germany) in a microwave oven for 6 min at 850 W followed by 2x 5 min at 600 W. The slides were then left in the buffer for 40 min at room temperature.

The IHC procedures were performed using Envision™ G/2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red; Dako, Glostrup, Denmark), however the staining for both antigens (Caspase-3 and Ki 67) was performed as single staining with Polymer/HRP (HRP conjugated Envision anti mouse/rabbit) as a secondary antibody and the color reaction was developed with 3, 3'- diaminobenzidine (DAB). The IHC procedures were performed following the first part of the protocol described by the manufacturer (staining with Polymer/HRP and DAB) with slightly modified incubation times.

In short, the slides were blocked with dual endogenous enzyme block for 5 min and incubated with the primary antibody (Ki-67 for 30 min at room temperature or caspase-3 overnight at 4⁰C) followed by secondary antibody (Polymer/HRP) for 30 min at room temperature. In between each step the slides were washed 2x 5 min with Tris buffered saline- TBS (50 mM Tris - Sigma, 150 mM NaCl, pH 7.6). The reaction was developed by incubation with DAB for 10 min. The slides were then washed with demineralized water and TBS, counterstained with Mayer's hematoxylin diluted 1:2 for 20 sec and mounted with glycerol (Dako, Glostrup, Denmark).

Microscopic evaluation

Light microscopic evaluation was performed blindly by the first author using 10x and 20x objectives. For each piglet two sections of the jejunum, two sections of the ileum and two sections of the colon were examined. Cells were scored as positive for Ki-67 when the nuclear staining was evident and for caspase-3 when the staining was present in the cytoplasm. The presence and localization of cells positive for proliferation and apoptosis markers in each group of piglets were described.

Quantitative analyses of proliferative cells were performed for each piglet. Ki-67 positive cells (cells with the nuclei stained brown by DAB) and a total number of cells determined by the sum of Ki-67 positive and negative cells (cells with the nuclei counterstained blue with hematoxylin) were counted in 20 vertically well oriented (with evident base and crypt-villus junction), randomly selected crypts in jejunum, ileum and colon. Only the cells that were lying next to each other on the top of the basement membrane were counted. The cells that were not visibly attached to the basement membrane were omitted.

Statistical analyses

Proliferation labeling indexes (LI) were calculated for each intestinal region in each piglet by dividing the total number of Ki-67 positive cells by the total number of cells counted from all scored crypts and multiplying by 100 ($\text{Ki-67 positive} / \text{total number of cells counted} \times 100$). Total number of cells, number of Ki-67 positive cells and LI were compared between the groups by t-test. Differences were considered significant at $p < 0.05$.

Results

Descriptive data

In all piglets the majority of Ki-67 positive cells were seen in the intestinal crypt epithelium in each region of the intestine and in lymphatic aggregates in Payer's patches in the ileum. Few Ki-67 positive cells were also seen in the lamina propria and tunica muscularis in each region of the intestine. The intensity of staining varied between the cells. No Ki-67 positive cells were observed in epithelium lining the villi in any of the piglet (Figures 1 and 2).

Apoptotic cells were seen rarely compared to the proliferative cells. Staining for caspase-3 was seen occasionally in the villus epithelium (range 1-5 cells on one villus) (Figure 3), in single cells in the lamina propria and the aggregates of the lymphatic tissue. In colon the staining for caspase-3 was seen sporadically in the surface epithelial cells and frequently in small particles present in varying numbers under and between the superficial enterocytes (Figure 4). Distribution of the apoptotic cells in piglets is summarized in Table 1.

Quantitative data

The results of quantitative analyses for proliferative cells are shown in Table 2.

The diarrheic piglets with villus atrophy had significantly higher number of crypt cells in the jejunum and cells positive for Ki-67 in the jejunum and ileum compared to the non-diarrheic piglets. Additionally, the number of Ki-67 positive cells was significantly higher in the jejunum of the diarrheic piglets with villus atrophy compared to the diarrheic piglets with normal villi. The diarrheic piglets with villus atrophy had

significantly higher LI in the jejunum compared to the piglets without villus atrophy and in the ileum compared to the non-diarrheic piglets. The diarrheic piglets with normal villi had higher total crypt cell, Ki-67 positive cells and LI in all intestinal regions in comparison to the non-diarrheic piglets; however except for total number of crypt cells in jejunum, the differences were not significant.

Discussions

This study investigated intestinal epithelial cell proliferation and apoptosis in the jejunum, ileum and colon of piglets selected from the herds affected by NNPDS.

Ki-67 staining with monoclonal mouse anti-human Ki-67 Antigen Clone MIB-1 and caspase-3 staining with Cleaved Caspase-3 (Asp175) antibody showed the expected staining pattern on the intestinal tissue.

Ki-67 positive cells were seen in the proliferative compartment in the crypt area and were not present either on the villi or in upper region of the colonic crypts. Approximately half of the cells present in the small intestinal crypts and one third in colonic crypts were shown to divide. High cell proliferation rates in neonatal animals are thought to be associated with intensive intestinal growth that takes place during the first days of life. It has been shown that in the jejunum of newborn pigs the mitotic indexes increase 40-50% within the first 1-2 days resulting in the 2-3 days cell renewal cycle [18, 19]. Therefore these relatively high cell proliferation rates seen in all piglets in this study were as expected.

The main finding of the present investigation was a statistically significant increase in the number of Ki-67 positive cells in the small intestinal crypts of the diarrheic piglets with pronounced villus atrophy. Expanded proliferation compartment is evidence for hyperplastic activity of the intestinal crypts and results in an increased number of crypt cells. These findings confirm that villus atrophy developed in the diarrheic piglets suffering from NNPDS is a type of a crypt hyperplastic villus atrophy associated with enlargement of the proliferative compartment. This condition is known to be a result of an increased rate of epithelial cell loss and is commonly seen in diseases that cause epithelial damage [11]. Due to epithelial destruction the mitotic activity of the crypt cells increases and new cells are rapidly produced in an attempt to compensate for increased cell loss. This results in intestinal crypt elongation. When cell loss rates exceed proliferation rates, the villi become atrophic. Evidently, the same pathophysiological mechanism occurs in NNPDS. No Ki-67 positive cells were seen in the villus epithelium in this study indicating that normal regulation of the proliferation was maintained and that maturation of the enterocytes occurred.

In the diarrheic piglets with normal villi the total number of crypt cells, Ki-67 positive cells and LI were lower compared to the piglets with atrophic villi but were slightly higher than those seen in the non-diarrheic piglets. Although the differences between these piglets and the non-diarrheic piglets were in general statistically not significant, those slightly higher values suggest that the epithelial cell turnover could also have been altered to a certain degree in these animals. All diarrheic piglets included in this study have had diarrhea for at least 2 days [14]. It is possible that these piglets were selected at the time of recovery when the pattern of cell proliferation was returning towards the normal range. If it is true, the lesions induced by the disease have potential to regress. Oppositely, these piglets could have been selected at the stage of the disease when the functional activity of the epithelium was already impaired but morphological changes in the mucosa were not yet developed. In this case, a slightly enhanced crypt proliferation could be result of the primary epithelial cell loss and precede villus atrophy. An additional

study on cell proliferation including larger number of animals at different stage of the disease would be more appropriate for both hypotheses testing.

In this study, the staining for caspase-3 was seen only sporadically in the epithelium on several villi in all piglets. Although apoptosis is thought to be a counterbalance to cell proliferation and in such rapidly divided tissue as intestinal epithelium a large number of apoptotic cells would be expected to be present, detection of apoptosis is limited. This is due to the rapidity of the process and immediate phagocytosis by macrophages and lymphocytes present underneath the epithelium [20, 21]. Moreover, it has been shown that caspase-3 independent mechanisms of cell programmed death also take place in the intestinal mucosa and the overall number of apoptotic cells detected by anti caspase-3 antibodies can be underestimated [22-26]. Thus the pattern of caspase-3 staining observed in this study was as expected. Caspase-3 positive cells were not seen in either crypt epithelium in the small intestine or in the proliferative compartment of the large intestine. It has been shown that in normal conditions stem cells also appear to undergo apoptosis and the total number of apoptotic cells in the small intestinal crypts is estimated to 10 % [19, 22-27]. However, our results are in agreement to other studies which previously have demonstrated an absence of apoptosis in the crypt stem cells [28, 29].

In a majority of the non-diarrheic piglets and the diarrheic piglets with normal villi only one or two apoptotic cells were occasionally present in the epithelium lining villus tips. In some piglets (five diarrheic with villus atrophy, two diarrheic with normal villi and three non-diarrheic) the apoptotic cells were also seen in the epithelium lining lower parts of the villi. Similar observations regarding the placement of apoptotic cells were reported by Godlewski *et al* in newborn piglets and chicken broilers [19, 30]. Thus the presence of apoptosis along entire length of the villi seems to be a characteristic feature in neonatal animal intestine. Nevertheless, in half of the diarrheic piglets with villus atrophy the apoptotic cells seemed to be more frequent (three-five cells on several villi). This slightly greater occurrence of apoptotic cells in atrophic intestine of the diarrheic piglets together with a significantly larger number of Ki-67 positive cells indicates that epithelial turnover rate was increased. However, it cannot be ruled out that slightly enhanced cell death was a primary event in the disease, as observed i.e. in *Salmonella* or certain strains of *E. coli* infections [9, 10, 13]. Alternatively the enhanced apoptosis could be a mechanism of elimination of infected enterocytes and initiate villus atrophy followed by cell proliferation in the crypts [31, 32].

We have previously reported elongation of the intestinal crypts in the colon of the diarrheic piglets [14]. It was expected to observe enhanced proliferation in the colonic epithelium that could have accounted for an increased depth of the colonic crypts. However, despite of the higher numbers of Ki-67 positive cells and labeling indexes in diarrheic piglets compared to non-diarrheic piglets, none of the values obtained by counting cells in the colonic mucosa was significantly different between the groups of piglets in this study. It seems that the mechanisms other than increased cell proliferation are responsible for deepening of colonic crypts in the course of NNPDs. However this finding should be further supported by additional studies since lack of statistical differences could be a result of a small number of animals included in this investigation. In similarity to other studies the number of apoptotic cells in the colonic epithelium was relatively small [24, 25]. Except for a few apoptotic cells present in the surface epithelium, caspase-3 staining was frequently seen in small particles located beneath the superficial epithelium in 21 piglets. These particles were most likely apoptotic bodies that are reported to be present in normal colonic

mucosa. Although accumulation of apoptotic bodies can be sometimes regarded as indication of minimal surface epithelium damage, their presence in almost all piglets included in this study regardless of the diarrheic status, suggests lesser importance of this finding.

Conclusions

Based on the results of this study, it can be confirmed that NNPDS induces alterations in intestinal epithelium turnover and villus atrophy with enlarged proliferative compartment. Increased mitotic activity in the intestinal crypts results most likely from increased epithelial cell loss. Furthermore, the results of this study suggest that the process of apoptosis may play role in the disease. In perspectives, in order to further identify mechanisms behind alterations in epithelial turnover with regards to identification of the etiology of NNPDS, additional studies on cell proliferation and death including a larger number of animals at different stage of infection should be implemented.

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Tables

Table 1. Distribution of apoptotic cells in piglets.

	No. of diarrheic piglets with villus atrophy (n=8)	No. of diarrheic piglets with normal villi (n=8)	No. of non-diarrheic piglets (n=8)
1-2 apoptotic cells at the top of several villi	3	6	6
3 or more apoptotic cells at the top of several villi	5	2	2
Apoptotic cells in lower parts of the villi	5	2	3
Apoptotic cells in luminal colonic epithelium	7	5	4
Staining for caspase-3 between and/or underneath luminal colonic enterocytes	6	7	8

Table 2. Quantitative data (mean \pm SD) on cell proliferation in piglets with and without villus atrophy (based on counting in 20 intestinal crypts).

		Diarrheic piglets with VA	Diarrheic piglets without VA	Non-diarrheic piglets
jejunum	Total cell no.	843.6 \pm 133.2 ^a	738.1 \pm 89.1 ^b	652.0 \pm 49.1 ^{ab}
	Ki-67 positive cell	508.4 \pm 98.3 ^{ab}	363.1 \pm 65.3 ^a	316.9 \pm 48.9 ^b
	LI	60.6 \pm 10.1 ^{ab}	49.2 \pm 7.5 ^a	48.2 \pm 7.0 ^b
Ileum	Total cell no.	760.9 \pm 92.2	741.4 \pm 54.2	696.4 \pm 60.7
	Ki-67 positive cell	419.6 \pm 73.9 ^a	388.9 \pm 70.7	330.9 \pm 60.4 ^a
	LI	54.9 \pm 6.4 ^a	50.6 \pm 9.5	47.1 \pm 5.8 ^a
Colon	Total cell no.	1315 \pm 97.7	1368 \pm 274.0	1222 \pm 181.3
	Ki-67 positive cell	463.6 \pm 113.9	415.3 \pm 140.5	358.8 \pm 93.3
	LI	34.9 \pm 6.9	30.3 \pm 7.1	29.7 \pm 8.1

^{a,b} Values labeled with the same letter differ significantly (p<0.05). LI- labeling indexes

Figures:

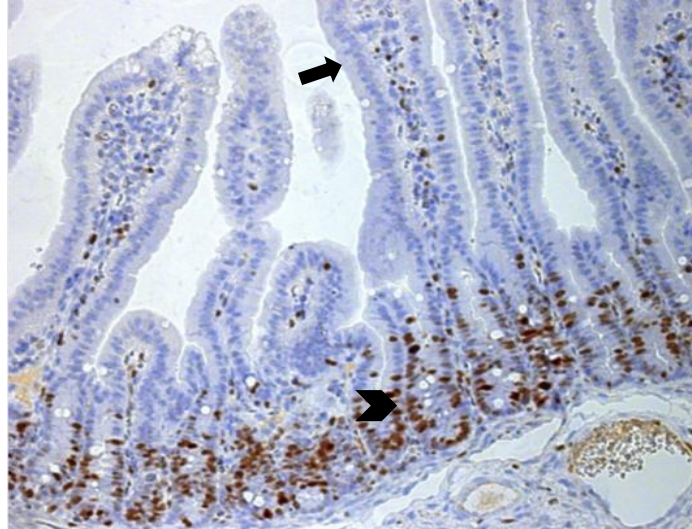


Figure 1. Immunohistochemical staining of proliferative cells in the small intestinal mucosa from the jejunum. Ki-67 positive cells (brown) are present in the epithelium lining the intestinal crypts (arrow head) and are absent in the epithelium lining the villi (arrow). Magnification 100x

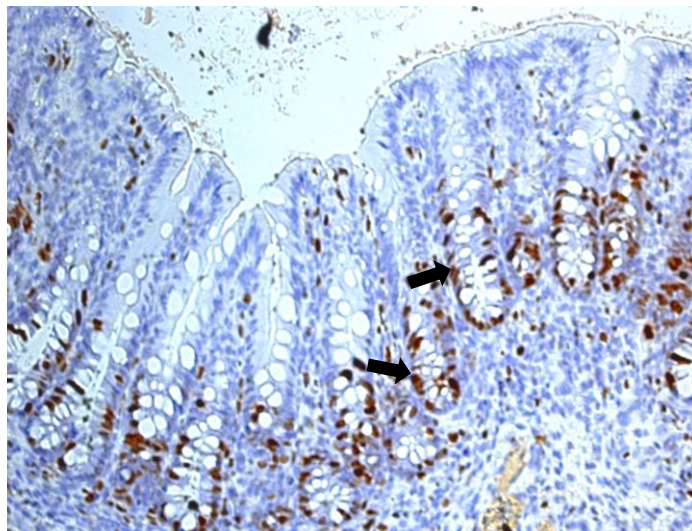


Figure 2. Immunohistochemical staining of proliferative cells in the intestinal mucosa from the colon. Ki-67 positive cells (brown) are present in the basal 1/3-1/2 of the crypt (arrows). Magnification 100x

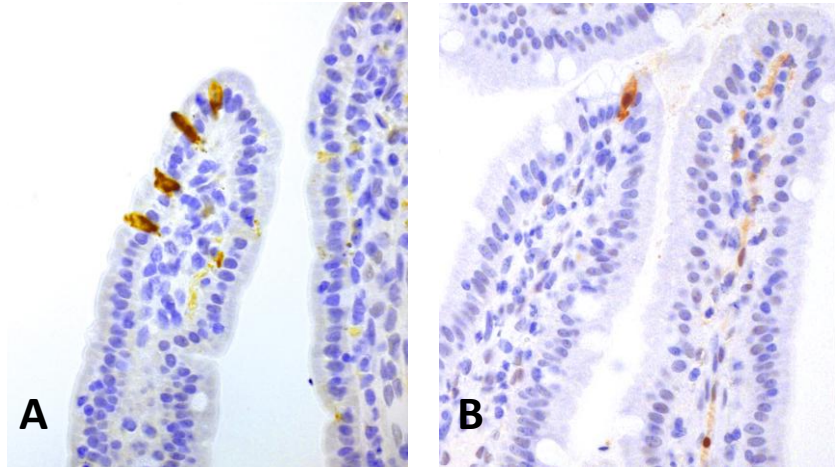


Figure 3. Immunohistochemical staining of apoptotic cells in the villus epithelium from the jejunum. **A.** One caspase-3 positive cell (brown) is present at the top of the villus in a non-diarrheic piglet. **B.** Four caspase-3 positive cells (brown) are present in the upper part of the villus in a diarrheic piglet. Magnification 200x

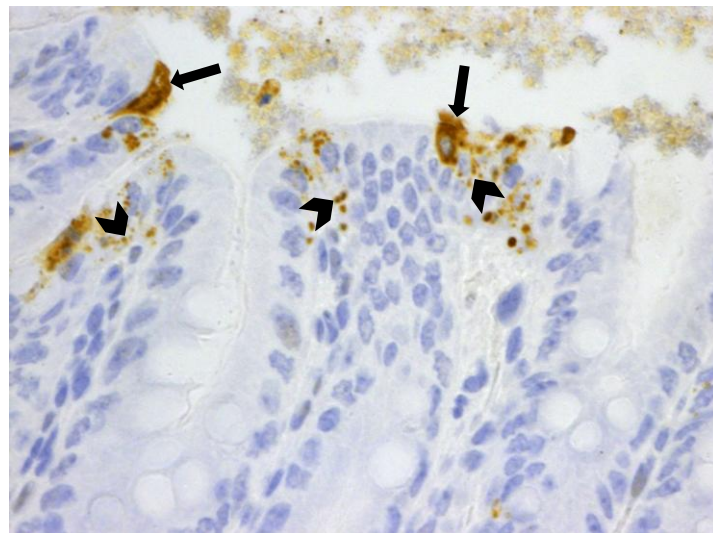


Figure 4. Immunohistochemical staining of apoptotic cells in the intestinal mucosa from the colon. Two caspase-3 positive cells are present in the luminal epithelium (arrows) and several apoptotic bodies are present between and underneath the epithelial cells (arrow heads). Magnification 200x.

5. Determination of the possible etiology of NNPDS

5.1. Preliminary microbiological field investigation

In this section, fluorescence in situ hybridization investigation, not presented in Manuscript III, is described. The aim of this study was to detect and identify bacteria colonizing the intestinal tissue of the piglets affected by NNPDS with respect to determination of their possible role in the etiology of the syndrome.

5.1.1. Detection of *Enterococcus durans*

Enterococcus durans (previously known as *Streptococcus durans*) is a Gram-positive, motile coccus-shaped bacterium occasionally present in the human and animal intestinal tract. Although Gram-positive cocci are believed to be commensal bacteria, *E. durans* has been occasionally associated with diarrhea in piglets, foals, calves and puppies [179-182]. A common feature of these infections was adherence of this bacterium to the intestinal villi. FISH investigation with the probe specific for *Enterococcus* spp. performed on tissue samples from the piglets affected by NNPDS has shown the presence of adherent enterococci in the small intestinal mucosa in 27% of the diarrheic piglets (see Manuscript III). This study was performed in order to determine whether *E. durans* was a member of the adherent enterococci community present in the intestinal tissue of the diarrheic piglets.

Materials and methods

In total ten diarrheic piglets positive for adherent *Enterococcus* spp. were selected for this study: two piglets from herd 1 (no. 9, 59), six piglets from herd 2 (no. 3, 9, 58, 77, 236, and 239) and two piglets from herd 3 (no. 12 and 115). Hybridization was carried out on the intestinal samples from duodenum (one transverse section), jejunum (two transverse sections), ileum (two transverse sections) and colon (two transverse sections). For each piglet a double hybridization with the general bacterial probe targeting 16SrRNA of *Domain bacteria* with the sequence 5'-GCTGCCTCCCGTAGGAGT-3 and labeled with fluorescein isothiocyanate (Eurofins MWG Operon, Ebersberg, Germany) and the specific *E. durans* probe targeting 23S rRNA with the sequence 5'-CTT ACT CGT GTA GAC AGA-3' labeled with a cyanine dye Cy3 (Eurofins MWG Operon, Ebersberg, Germany) was performed. Hybridization was carried out according to the protocol described in Appendix B.

Microscopic investigation was performed by the author of this thesis using a Zeiss Axioimager M1 epifluorescence microscope equipped with a 120-W HBO lamp (Carl Zeiss, Oberkochen, Germany) under magnification 40x. For detection of fluorescence signals the following filter sets were applied: filter set 38 for detection of fluorescein, filter set 43 for detection of Cy3 and filter set 24 for simultaneous detection of green and red fluorescence. The positive score was given when the hybridization signals were clearly specific and distinguishable as bacterial cells and could be identified with the specific probe.

Results

Fluorescence signals for *E. durans* were seen in the ileum and colon of the piglet no. 9 from herd 1. Small amounts of bacteria were present in the intestinal lumen forming microcolonies. No adherence of *E. durans* to the intestinal epithelium was seen in this piglet. There were no fluorescence signals for *E. durans* in piglets no. 3, 9 (from herd 2) 12, 58, 59, 77, 115, 236, and 239.

5.1.2. Detection of *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* in experimentally infected piglets

This study was performed on the intestinal tissue samples from 13 piglets with experimentally induced NNPDs (litter 2; see Manuscript IV). Histopathological investigation performed on the tissue samples from these piglets has revealed the presence of bacteria in the small intestinal lumen, between the villi and in close contact to the luminal surface of the villus epithelium. The aim of this study was to detect *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* in the intestinal mucosa of these piglets.

Material and methods

Animals

This study was performed on the intestinal tissue samples from the piglets experimentally challenged with inoculum derived from diarrheic piglets collected from herd 1. For description of the animals and experimental infection the reader is referred to the Manuscript IV. The piglets included in this study are referred to as litter 2 in the manuscript.

FISH

Hybridization was carried out on the intestinal samples from jejunum ileum and colon from 13 piglets (9 inoculated and 4 non-inoculated). For each piglet a double hybridization with the general bacterial probe targeting 16S rRNA of *Domain bacteria* labeled with fluorescein isothiocyanate and one of the specific probes targeting 16S or 23S rRNA for *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* labeled with a cyanine dye Cy3 (Eurofins MWG Operon, Ebersberg, Germany) was performed. Probes sequences and hybridization protocol are described in Appendix B.

Microscopic investigation was performed by the author of this thesis using a Zeiss Axioimager M1 epifluorescence microscope equipped with a 120-W HBO lamp (Carl Zeiss, Oberkochen, Germany) under magnification 40x. For each animal, three transverse sections of the jejunum, two transverse sections of the ileum and one transverse section of the colon was examined. For detection of fluorescence signals the following filter sets were applied: filter set 38 for detection of fluorescein, filter set 43 for detection of Cy3 and filter set 24 for simultaneous detection of green and red fluorescence. Micrographs were taken using an AxioCam MRm version 3 FireWire monochrome camera and AxioVision software, version 4,5 (Carl Zeiss, Oberkochen, Germany). The positive score was given when the hybridization signals were clearly specific and distinguishable as bacterial cells and could be identified with the specific probe.

Results

Fluorescence signals for *Domain Bacteria* were seen in all tissue specimens from all piglets. Bacteria were present in the intestinal lumen and space between the villi. Additionally in eight piglets (six inoculated and two non-inoculated) bacterial cells adhered to the epithelium lining the villi in the jejunum and ileum.

E. coli was detected in all tissue specimens from all piglets. Adherent *E. coli* was present in the small intestinal mucosa in six inoculated and two non-inoculated piglets (Figure 15A). In the remaining piglets *E. coli* were present in the intestinal lumen and space between the villi.

Nine piglets were positive for *Enterococcus* spp. In one non-inoculated piglet the fluorescence signals for enterococci were present only in the intestinal lumen. In the remaining eight piglets (six inoculated and two non-inoculated) *Enterococcus* spp. were found in close proximity and adhered to the intestinal epithelium lining the villi in the jejunum and ileum (Figure 15B). These were the piglets in which the small intestinal mucosa was simultaneously colonized by adherent *E. coli*.

Fluorescence signal for *C. perfringens* were seen in the intestinal lumen of three piglets (two inoculated and one non-inoculated). *C. difficile* was present in the colon of three piglets (one inoculated and two non-inoculated).

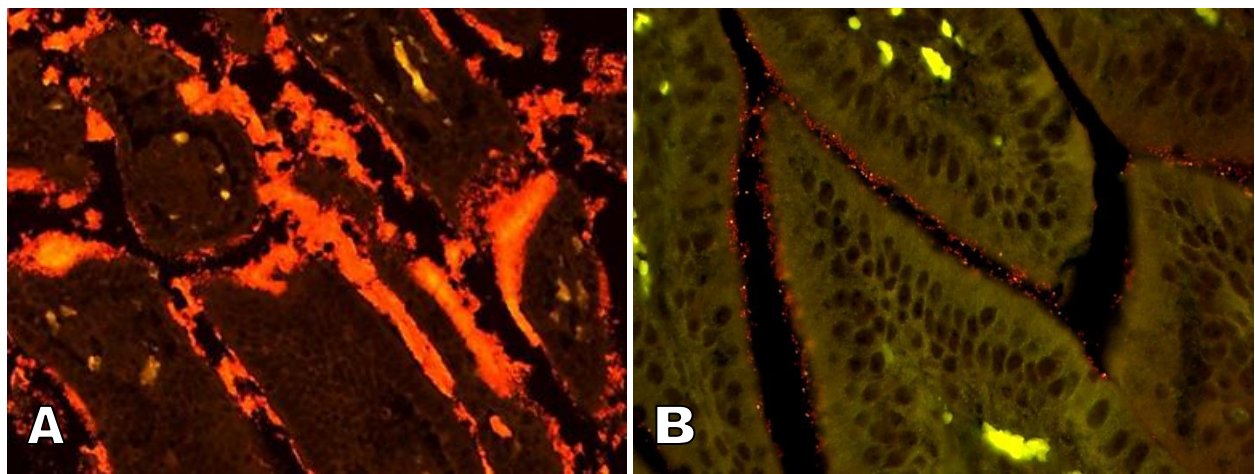


Figure 15. Tissue sections from the jejunum of experimental piglets from litter 2.

A. Hybridization for *E. coli* (red), magnification 200x.

B. Hybridization for *Enterococcus* spp. (red), magnification 400x.

5.2. Manuscript III

Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea

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RESEARCH ARTICLE

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Fluorescence in situ hybridization investigation of potentially pathogenic bacteria involved in neonatal porcine diarrhea

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Abstract

Background: Neonatal diarrhea is a multifactorial condition commonly present on pig farms and leads to economic losses due to increased morbidity and mortality of piglets. Immature immune system and lack of fully established microbiota at birth predispose neonatal piglets to infection with enteric pathogens. The microorganisms that for decades have been associated with enteritis and diarrhea in suckling piglets are: rotavirus A, coronavirus, enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens* type C, *Cryptosporidium* spp., *Giardia* spp., *Cystoisospora suis* and *Strongyloides ransomi*. However, in recent years, the pig industry has experienced an increased number of neonatal diarrhea cases in which the above mentioned pathogens are no longer detected. Potentially pathogenic bacteria have recently received focus in the research on the possible etiology of neonatal diarrhea not caused by common pathogens. The primary aim of this study was to investigate the role of *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* in the pathogenesis of neonatal porcine diarrhea with no established casual agents. Fluorescence in situ hybridization with oligonucleotide probes was applied on the fixed intestinal tissue samples from 51 diarrheic and 50 non-diarrheic piglets collected from four Danish farms during outbreaks of neonatal diarrhea not caused by well-known enteric pathogens. Furthermore, an association between the presence of these bacteria and histological lesions was evaluated.

Results: The prevalence of fluorescence signals specific for *E. coli*, *C. perfringens* and *C. difficile* was similar in both groups of piglets. However, *Enterococcus* spp. was primarily detected in the diarrheic piglets. Furthermore, adherent bacteria were detected in 37 % diarrheic and 14 % non-diarrheic piglets. These bacteria were identified as *E. coli* and *Enterococcus* spp. and their presence in the intestinal mucosa was associated with histopathological changes.

Conclusions: The results of this study showed that simultaneous colonization of the intestinal mucosa by adherent non-ETEC *E. coli* and *Enterococcus* spp. can be involved in the pathogenesis of neonatal porcine diarrhea. These bacteria should be considered in diagnosis of diarrhea in piglets, when detection of common, well-known enteric agents is unsuccessful.

Keywords: *E. coli*, *Enterococcus*, Fluorescence in situ hybridization, Neonatal diarrhea, Potentially pathogenic bacteria

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Background

Neonatal diarrhea is a major problem that leads to reduced weight gain, increased morbidity and mortality among piglets and has, in consequence, an economic impact on the swine industry. Diarrhea in suckling piglets is often of infectious nature and results from interactions between pathogens, an immature immune system and the environment. In the past, a number of microorganisms have been associated with pig neonatal enteritis i.e. rotavirus A, coronavirus, enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens* type C, *Cryptosporidium* spp., *Giardia* spp., *Cystoisospora suis* and *Strongyloides ransomi* [1,2]. However, in recent years, the pig industry in many countries has experienced an increased number of neonatal diarrhea cases not caused by the above mentioned pathogens [3-5]. Considering contribution of bacterial agents, identification of the etiology of this diarrhea becomes challenging and problematic. The gastrointestinal tract hosts diverse population of microorganisms composed of commensals and potentially pathogenic bacteria. These bacteria interact constantly in a synergistic and competitive manner and any disturbances in this microbial community can cause bacterial overgrowth and disease [6]. Due to immunodeficiency and incomplete gut microbiota at birth, the neonatal piglets are particularly vulnerable to infectious enteric diseases [7]. However, recognition of the pathogenic bacteria in gut population and distinguishing from non-harmful microbiota is difficult and restricted by the fact that bacterial diversity is not yet fully explored and the pathogenic bacteria are also present in clinically healthy animals. Nevertheless, a few potentially pathogenic bacteria that are considered to be part of the normal intestinal microbiota, have been recently studied as a potential cause of neonatal enteritis. The aim of this study was to investigate the role of the following bacteria: *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* in naturally occurring pig neonatal diarrhea not caused by known enteric pathogens. For detection of these bacteria we used fluorescence in situ hybridization (FISH) targeting ribosomal RNA as this is a rapid and reliable method that allows a direct visualization, identification and spatial localization of bacteria within fixed tissue samples [8,9].

Materials and methods

Animals

In total 51 diarrheic and 50 non-diarrheic piglets aged 3–7 days were included in this study. The piglets were selected from four commercial Danish swine herds presenting high standards of management and housing. Diarrhea of unknown etiology (not caused by either enterotoxigenic *E. coli*, *C. perfringens* type C, rotavirus A, coronavirus or parasites) and poorly responding to

antibiotic therapy was present in at least 30 % litters in each herd for a period of minimum six months. From each herd 11–14 diarrheic and 12–13 non-diarrheic piglets (age matched) from several litters (maximum two piglets per litter) were selected. The diarrheic piglets had diarrhea for at least 2 days prior to euthanasia and were selected from the litters with the highest prevalence of diarrhea. The non-diarrheic piglets did not have diarrhea at any time and were selected from the litters with no diarrhea or very low prevalence of diarrhea. For further details on the selection of herds and piglets the reader is referred to Kongsted et al.; 2013 [3].

Intestinal samples and histopathology

The samples of duodenum, jejunum, ileum and spiral colon were collected during necropsies, fixed in 10 % neutral buffered formalin for at least 48 h, embedded in paraffin wax and cut at 3 μ m. From each piglet one section of duodenum, two sections of jejunum, two sections of ileum and two sections of colon were examined histopathologically and by FISH. Sections for histopathology were stained with hematoxylin and eosin (HE) according to standard laboratory procedures and investigated microscopically for the presence of histological lesions, which is described in Kongsted et al. [3]. Sections for FISH were mounted on SuperFrost®White slides (Mensel-Gläser, Braunschweig, Germany). Prior to hybridization the sections were deparaffinized in xylene (2 \times 2 min), treated with 99.9 % ethanol (2 \times 2 min) and air dried.

Fluorescence in situ hybridization (FISH)

FISH was carried out with oligonucleotide probes targeting bacterial 16S or 23S rRNA as listed in Table 1. For each animal FISH was performed as double hybridization with two probes: a general bacterial probe targeting *Domain bacteria* (labeled with fluorescein isothiocyanate) and one of the specific probes directed against *E. coli*, *Enterococcus* spp., *C. perfringens* or *C. difficile* (labeled with the cyanine dye Cy3).

Hybridization was carried out overnight with each probe in concentration of 5 ng/ μ l hybridization buffer (1 M Tris- pH 7.2, 5 M NaCl, 10 % sodium dodecyl sulphate) at 45 °C. The sections were then washed 3 \times 3 min in pre-warmed (45 °C) hybridization buffer and 3 \times 3 min in pre-warmed (45 °C) washing buffer (1 M Tris- pH 7.2, 5 M NaCl). The samples were finally rinsed in water, air dried and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA) for epifluorescence microscopy.

Epifluorescence microscopy

Microscopic examination was performed using a Zeiss Axioimager M1 epifluorescence microscope equipped with a 120-W HBO lamp. The following filter sets were applied for detection of fluorescence signals: filter set 38

Table 1 List of the oligonucleotide probes used in this study*

Target bacteria	Name of the probe	Target sequence (5'-3')	Target region of rRNA	References
<i>Domain bacteria</i>	S-D-Eub-0338	5'-GCTGCCTCCCGTAGGAGT-3'	16S	[10]
<i>Escherichia coli</i>	S-S-E.coli-1161	5'-GCATAAGCGTCGCTGCCG-3'	23S	[11]
<i>Enterococcus spp.</i>	S-G-Enteroco-184	5'-CAAATCAAACCATGCGG-3'	16S	[12]
<i>Clostridium perfringens</i>	S-S-C.perfring-1	5'-TGTTGAATGATGATGCC-3'	16S	[13]
<i>Clostridium difficile</i>	S-S-C.diff-193	5'-TGTAAGGCTCACCTTG-3'	16S	[14]

* The probes were purchased from Eurofins MWG Operon, Ebersberg, Germany.

for detection of fluorescein, filter set 43 for detection of Cy3 and filter set 24 for simultaneous detection of green and red fluorescence. Micrographs were taken using an AxioCam MRm version 3 FireWire monochrome camera and AxioVision software, version 4.5 (Carl Zeiss, Oberkochen, Germany).

Evaluation of fluorescence signals

Evaluation of fluorescence signals in all intestinal regions from each piglet was performed blindly by the first author using a 40× objective and included determination of the amount of bacterial cells and their distribution in the tissue section. A positive score for particular bacteria was given when the hybridization signals were clearly specific and distinguishable as bacterial cells and could be identified with the specific probe. The amount of bacteria in each intestinal region was scored in a semi-quantitative manner according to the following scale: + small amount, ++ moderate amount, +++ large amount of bacteria. A score of + was given to the tissue specimens in which the number of bacterial cells present in the microscopic field was in a countable range and/or bacterial cells were clustered in small groups (Figure 1A). When amount of bacterial cells was out of a countable range and the bacterial cells were grouped over larger areas of the microscopic field, the tissue specimen received ++ (Figure 1B). A score of +++ was given to the tissue specimens in which bacteria were present in a strikingly large amount as well as when single bacterial cells could no longer be distinguished due to high concentration of bacteria and the fluorescence signals were fused together (Figure 1C). Evaluation of the total amount of bacteria in the small intestines was based on the highest score given for a particular intestinal region.

Statistical analyses

Presence and localization of the investigated bacteria data were modeled with a mixed effects model, where the logit of the probability of bacterial presence and location was allowed to depend on diarrheic status, presence of histological lesions and age of the piglets. Herd was included as a random effect. Spearman rank correlation was calculated between the presence of pathogens and histological lesions. Furthermore, the amounts of

the bacteria were analyzed with a multinomial effects model where herd, diarrheic status, age and presence of histological lesions were explanatory variables, using neural networks for parameter estimation. Model reduction was performed using the likelihood ratio method.

Results

Hybridization with the universal probe targeting *Domain bacteria* showed bacteria of various morphotypes in all piglets. In vast majority of piglets the largest amounts of bacteria were present within the intestinal content in the colon. However, if the content was missed during tissue preparation, the amounts of bacteria seen in the colon were lower compared to other regions of the intestine. In the small intestines larger amounts of bacteria were usually seen in the ileum compared to the jejunum and duodenum. In the latter one the bacterial cells were present only in small amounts in all animals. Bacteria were spread in the intestinal lumen and the space between the villi. Additionally, in 37 % of diarrheic and 14 % of non-diarrheic piglets bacterial cells adhered to the intestinal epithelium lining the villi (Figure 2). There was no specific fluorescence seen in intracellular regions, however, in two diarrheic piglets with diffuse necrotic changes in the mucosa the fluorescence signals for *Domain bacteria* were seen in large amounts within the necrotic tissue.

In the small intestines positive signals for *E. coli* were seen in 88 % of diarrheic and 80 % of non-diarrheic piglets. *E. coli* were present mostly in the intestinal lumen and luminal space between the villi. The diarrheic piglets had more frequently large amounts of *E. coli* compared to the non-diarrheic piglets ($p < 0.05$). Additionally, in 33 % of diarrheic and 14 % of non-diarrheic piglets *E. coli* adhered to the villous epithelial cells (Figure 3). The majority of the diarrheic piglets (59 %) with adherent *E. coli* originated from one herd. The presence of *E. coli* was positively correlated with villous atrophy (r_s : 0.20, $p < 0.05$) and neutrophil infiltration (r_s : 0.20, $p < 0.05$) regardless of the diarrheic status.

In the colon positive fluorescence signals for *E. coli* were present within the intestinal content or in the lumen in all piglets. Additionally, in two diarrheic piglets the bacteria adhered to the luminal surface of the

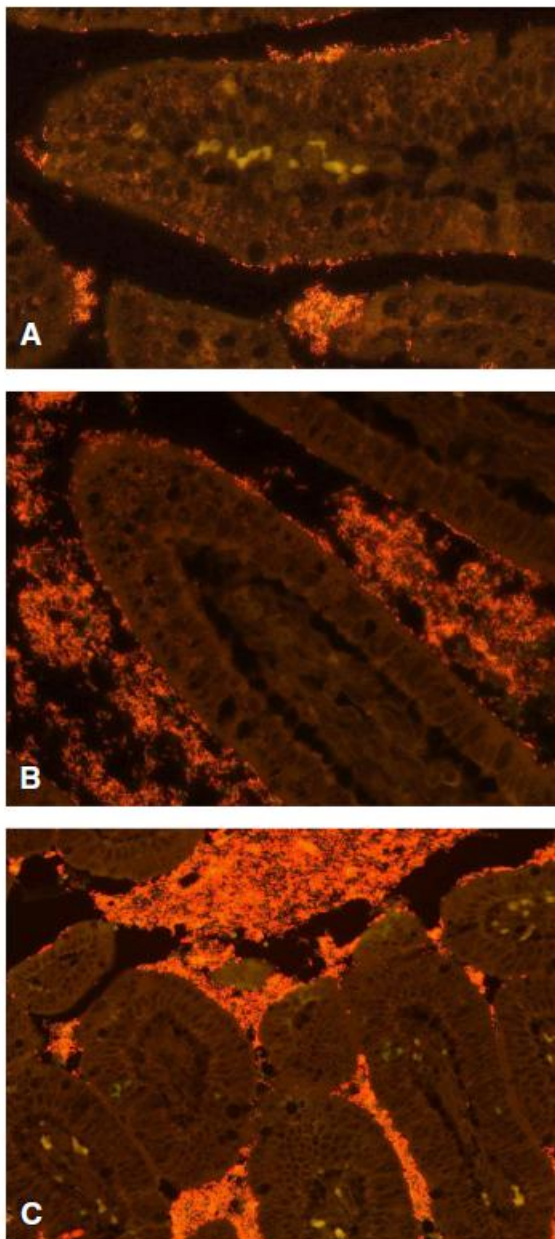


Figure 1 Representative micrographs showing semi-quantitative scores of the fluorescence signals in the microscopic field. **A.** + small amount of bacteria (red), magnification 400x. **B.** ++ moderate amount of bacteria (red), magnification 400x. **C.** +++ large amount of bacteria (red), magnification 400x.

colonic epithelium that showed excessive extrusion and deformation of the enterocytes and in one non-diarrheic piglet *E. coli* adhered to the luminal colonic enterocytes that otherwise appeared normal.

Enterococcus spp. cells were detected in the small intestines of 45 % of diarrheic and 8 % of non-diarrheic piglets and their presence was positively correlated with

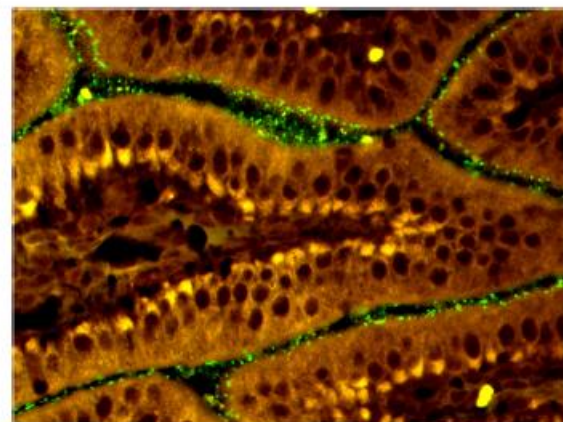


Figure 2 Section of the jejunum from a diarrheic piglet hybridized with the general bacterial probe. Mixed colony of cocci and coliform bacteria (green) intimately adhere to the villous epithelial cells (brown). Magnification 400x.

villous atrophy ($r_s: 0.26, p < 0.01$). Adherent enterococci were seen in association with the small intestinal epithelium lining the villi in 27 % of diarrheic and 2 % of non-diarrheic piglets (Figure 4). In the majority of cases adherent enterococci were seen in the small intestine of those diarrheic piglets that also had adherent *E. coli* ($r_s: 0.58, p < 0.0001$). Presence of adherent enterococci was associated with mild epithelial lesions ($r_s: 0.45, p < 0.05$). In the piglets aged 3 days the presence of adherent enterococci was positively correlated with neutrophil granulocyte infiltration, however the correlation was negative in the piglets aged 4–7 days.

Positive fluorescence signals for *C. perfringens* were detected in 73 % of diarrheic and 78 % of non-diarrheic piglets. These bacteria were mostly present in the intestinal lumen, however, in 20 % of diarrheic and 30 % of non-diarrheic animals the bacteria were found within the mucus layer and in direct contact with the intestinal epithelium. Additionally, in the ileum and colon of one diarrheic piglet *C. perfringens* cells were seen in large amounts within the necrotic tissue alongside other bacteria detected by the general bacterial probe. Otherwise there was no significant correlation between the presence and location of *C. perfringens* in the intestinal tissue and histological lesions. *C. difficile* cells were detected in small amounts within the intestinal content of the colon in 65 % of diarrheic and 70 % of non-diarrheic piglets. Single *C. difficile* cells were also present in the lumen of the small intestine in 12 % of diarrheic and 14 % of non-diarrheic piglets. There was no correlation between the presence of *C. difficile* and histological lesions and diarrhea.

The results of semi-quantitative score are shown in Figure 5.

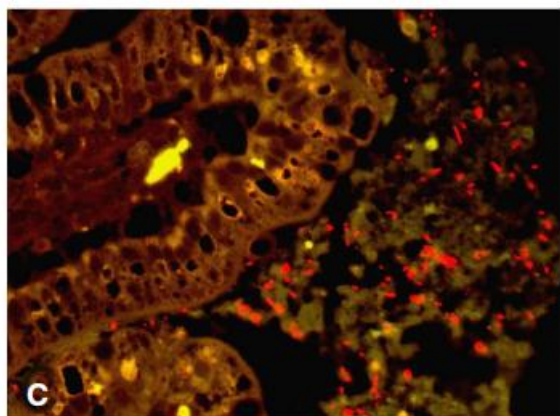
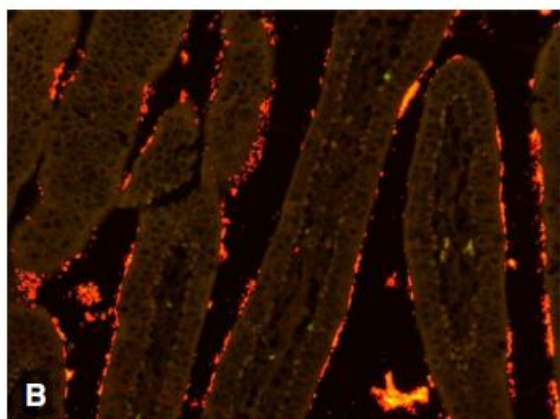
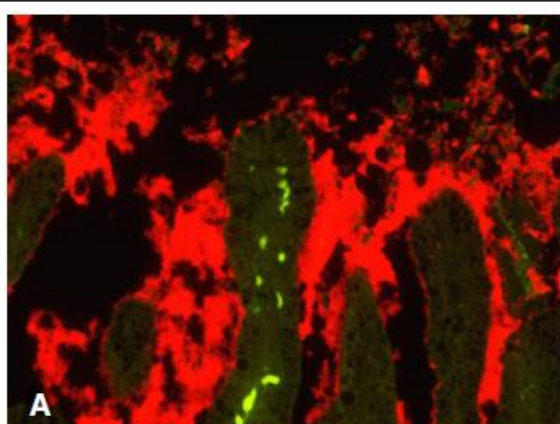


Figure 3 Intestinal tissue sections colonized by *E. coli*. **A.** Jejunum from a diarrhetic piglet. Large fluorescence mass consisting of *E. coli* (red) present in the intestinal lumen, between the villi (green) and attached to the villous epithelium. The yellowish-green fluorescence present in the centrally located villous results from autofluorescence of the erythrocytes. Magnification 200x. **B.** Jejunum from a diarrhetic piglet. *E. coli* cells (red) adhere to the intestinal epithelium lining the villi (brown). Magnification 200x. **C.** Jejunum of a non-diarhetic piglet. Small amounts of *E. coli* are present in the intestinal lumen with no adhesion to the tissue. Magnification 400x.

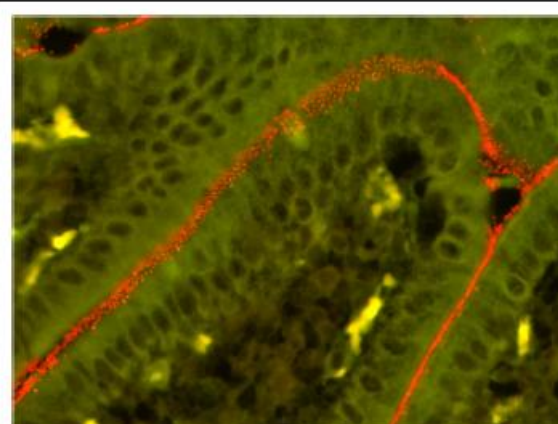


Figure 4 Section of the jejunum from a diarrhetic piglet hybridized with the probe specific for *Enterococcus* spp. Enterococci (red) adhere to the epithelium lining the villi (greenish). Magnification 400x.

Discussion

The objective of this study was to elucidate the role of *E. coli*, *Enterococcus* spp., *C. perfringens* and *C. difficile* in neonatal porcine diarrhea with no previously established etiology. We used FISH method for investigation of the prevalence, abundance and location of these potentially pathogenic bacteria in the intestinal tissue because a direct visualization of microorganisms helps to determine their association with the mucosa surface and may therefore have a potential value in elucidating their role in the disease. Sensitivity of FISH method for detection of bacteria depends on many factors including metabolic activity of microbial cells and availability of target sequences in the tissue samples. It has been shown that in natural samples the fluorescence signal intensity may be too low for identification of microorganisms [15]. However, because the intestinal microbiota are expected to express high metabolic activity and possess high content of rRNA, the FISH method seems to be an appropriate approach for in situ detection of enteric bacteria. In order to better control for eventual false negative results due to methodological problems we decided to apply a general bacterial probe simultaneously with the specific probe on all tissue specimens. As the FISH results of hybridization with the general probe were satisfactory, we consider sensitivity of FISH performed in this investigation to be high.

According to informations provided at probeBase [16], three oligonucleotide probes used in this study: *Enterococcus* spp, *C. perfringens* and *C. difficile* probes, were shown to be highly specific and reliable for detection of these particular bacteria. However, the *E. coli* probe used in this investigation was noted to be unable to target all *E. coli* strains. Additionally, the target sequence of this

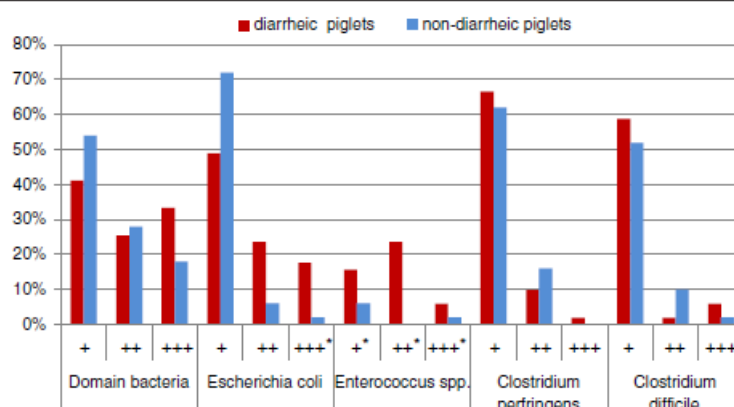


Figure 5 Semi-quantitative analyses of the fluorescence signals in the intestines of 51 diarrheic and 50 non-diarrheic piglets. +: Small amounts, ++: moderate amounts, +++: large amounts of bacteria. *depicts significant positive correlation between amount of bacteria and diarrhea (p-value <0.05).

probe was shown to match other enterobacteria; however these were not relevant as swine pathogens.

Quantification of microbial community detected by FISH in the intestinal tissue samples is not possible when the bacteria are present in high concentration as it hinders distinction of fluorescence signals from single bacterial cell. Therefore, evaluation of the amount of bacteria was done in a semi-quantitative manner, based on the subjective judgment of the investigator.

Adhesion of bacteria to the epithelial cells is believed to be an initial and pivotal event in the pathogenesis of most bacterial enteric infections and is necessary for allowing bacteria to survive and persist in a continuously moving environment and to defeat host defense mechanisms [6]. In this study adherent bacteria were seen in 37 % of diarrheic and 14 % of non-diarrheic piglets. The non-diarrheic piglets were collected from the same herds as the diarrheic ones. Therefore, it cannot be excluded that some of the non-diarrheic piglets were in the initial phase of infection, thus expressed similar composition of eventual pathogens as the diarrheic piglets. In situ hybridization with the specific probes identified these bacteria as *E. coli* and/or *Enterococcus* spp. and there was a significant positive correlation between adherence of these bacteria and diarrhea. Furthermore, large amounts of *E. coli* were seen in significantly higher number of diarrheic piglets compared to non-diarrheic animals. Overgrowth and colonization of the mucosal surface by *E. coli* suggest its involvement in diarrhea. The piglets involved in this study were negative for enterotoxigenic *E. coli* (ETEC) [3], which are considered to be the most common cause of pig neonatal diarrhea and for which the ability to attach to and colonize the intestinal epithelium is believed to be a hallmark virulence trait [17]. However, *E. coli* strains other than ETEC have also been shown to be able to adhere to the intestinal mucosa surface and cause

diarrhea [18]. In addition, attaching and effacing *E. coli* (AEEC) that have ability to cause attaching and effacing lesions in the gut mucosa, have been associated with diarrhea in domestic animals including pigs [19,20]. Therefore, further work will be done towards identifying and defining the pathogenicity of adherent *E. coli* found in this study.

We observed a significant positive correlation between the presence of *E. coli* and histomorphological changes in the intestinal mucosa. Villous atrophy is a common condition in diarrheal diseases and in suckling piglets this is primarily associated with viral or parasitic infections [21]. However, some reports have shown that shortening of villi and epithelial lesions can follow colonization of the mucosa by *E. coli* [22,23]. Since the piglets included in this study were thought to be free from infection with commonly known pathogens, at this stage of investigation it is difficult to conclude whether overgrowth and colonization of the mucosa by *E. coli* was a primary event in the pathogenesis of villous atrophy or was secondary to infection with other, yet unidentified microorganisms, which cause alteration in the intestinal villi. Further studies are currently being conducted in order to determine the etiology of the presently described diarrhea.

Enterococci are commensal bacteria in the intestinal tract. However, it has been reported that certain members of enterococci can sporadically cause diarrhea in neonatal animals including piglets [24-27]. The pathogenic potential of enterococci seems to be associated with their ability to intimately adhere to the intestinal epithelium but the mechanisms by which these bacteria cause diarrhea remain unclear. So far, no evident mucosal damage has been reported in association with enterococci infection. In this study we also observed adhesion of *Enterococcus* spp. to the intestinal epithelial cells in the diarrheic piglets, which suggests pathogenic ability

of these bacteria. A significant positive correlation between the presence of enterococci and histological lesions (villous atrophy and mild epithelial lesions) can be explained by the fact that the positive fluorescence signals for adherent *Enterococcus* spp. were seen in the small intestine of piglets that also had adherent *E. coli*. If these lesions were a consequence of a bacterial infection, they should be associated with *E. coli* rather than *Enterococcus* spp. as discussed above. Nevertheless, simultaneous colonization of the intestinal mucosa surface by these bacteria is an interesting finding and suggests their close interactions. Previously, a virulent synergistic effect between *E. coli* and *E. faecalis* has been described in relation to experimental polymicrobial infections [28] and it has been suggested that *E. faecalis* may inhibit phagocytosis of other pathogens including *E. coli*, and prevent them from intracellular death [29].

The majority of the piglets positive for adherent *E. coli* and adherent enterococci belonged to the same herd, which indicates that environmental factors influence composition of intestinal microbiota and eventual pathogens. This finding emphasizes the complexity of pathogenesis of porcine neonatal diarrhea and suggests that consideration of herd related aspects may be crucial for diagnosis and control of diarrheic conditions in piglets.

C. perfringens type A and *C. difficile* are nowadays regarded as ones of the most common bacterial species involved in pig neonatal diarrhea worldwide [30]. In this study, the occurrence of *C. perfringens* and its amount detected by FISH were similar in diarrheic and non-diarrheic piglets. Pathologically, degenerative and necrotic changes in the intestinal mucosa are commonly associated with clostridial enteritis and the bacteria are usually present among the necrotic tissue [30]. Such lesions were observed only in one diarrheic piglet in this study and it has been confirmed by microbiological testing that this piglet was positive for *C. perfringens* type C [3], which in that case can be regarded as a cause of enteritis. However, the mechanisms that could be involved in *C. perfringens* type A infection, remain unclear and there is no certain evidence for an adhesion of this bacterium to not destroyed intestinal tissue. Only few studies have investigated *C. perfringens* type A adhesive properties [31,32], but their results were inconclusive and to date, it is generally believed that *C. perfringens* does not have the ability to adhere to healthy intestinal epithelium. In agreement with this, the presence of *C. perfringens* in close proximity to the mucosal surface was seen in similar prevalence in both groups of piglets in this study (20 % diarrheic vs. 30 % non-diarrheic) and did not correlate with histological lesions, suggesting that the localization of *C. perfringens* cells in the intestinal mucosa is not linked to its pathogenicity. However, the pathogenesis of clostridial enteritis is commonly associated with the ability to

produce toxins and diagnosis of the infection is based on the detection of large numbers of toxigenic bacteria [30]. Therefore, the role of *C. perfringens* type A should not be definitely ruled out and the determination of the importance of this bacterium in neonatal diarrhea should be supported by thorough investigation on clostridial toxins.

C. difficile infections are currently reported as one of the most common causes of pig neonatal diarrhea in some countries and whenever diagnosed, the culturing reveals heavy growth of this bacterium [33]. Microscopically, *C. difficile* infection is characterized by catarrhal, fibrinous or purulent colitis, however such lesions were not observed in this study. Furthermore, there was no association between the presence of this bacteria and pathological changes in the colon. Moreover, the occurrence of *C. difficile* and its amount did not differ significantly between diarrheic and non-diarrheic piglets. Therefore the presence of this bacterium seems not to be linked to the investigated diarrhea and these results are in agreement with other reports [34]. Additional studies with focus on clostridial toxins are being conducted to determine the role of both *Clostridia* species in the pathogenesis of presently reported neonatal diarrhea.

Conclusions

Based on the results of this study, we conclude that potentially pathogenic bacteria such as non-ETEC *E. coli* and *Enterococcus* spp. might be involved in neonatal diarrhea in pigs less than 1 week old. Further identification of these bacteria is necessary in order to determine their pathogenicity and role in this syndrome. These bacteria should be taken into consideration whenever diagnosis of the pathogens commonly associated with neonatal porcine diarrhea fails.

Animal ethics

The study was conducted in compliance with general ethical principles and with informed client consent. All farms providing animals presented high standards of veterinary care.

Competing interests

The authors of this research paper have no competing interests.

Authors' contributions

BJ, MB and TKJ participated in the conception and design of this study and contributed to the interpretation of the results. Histopathological and FISH investigation and drafting of the manuscript were performed by BJ. MB and TKJ revised the manuscript for intellectual content. AS performed statistical analyses. All authors participated in proofreading and approval of the final manuscript.

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5.3. Manuscript IV

Experimental reproduction of clinical symptoms and histological lesions characteristic of new neonatal porcine diarrhea syndrome in newborn piglets

Beata Jonach, Øystein Angen, Hanne Kongsted, Tim Kåre Jensen

Submitted to: BMC Veterinary Research (2014)

Experimental reproduction of clinical symptoms and histological lesions characteristic of new neonatal porcine diarrhea syndrome in newborn piglets

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Abstract

Background

In recent years an increased number of neonatal porcine diarrhea cases with unknown etiology have been reported from several countries giving rise to speculation that a new disease has emerged. A comprehensive study on the pathogenesis of this diarrheal syndrome, referred to as New Neonatal Porcine Diarrhea Syndrome (NNPDS), has been conducted on four Danish pig farms. That study showed that none of the common enteric pathogens; rotavirus A, coronavirus, enterotoxigenic *Escherichia coli*, *Clostridium perfringens* type A and C, *Clostridium difficile*, *Cryptosporidium* spp., *Giardia* spp., *Cystoisospora suis* and *Strongyloides ransomi* were the cause of this diarrhea; however the exact etiology has not yet been determined. The aim of this study was to establish an animal model of New Neonatal Porcine Diarrhea Syndrome in newborn piglets in order to enable further investigations on the pathogenesis. Ten litters of piglets were orally challenged with either the homogenized intestinal tissue or deprived of bacteria filtrate of the intestinal tissue homogenate derived from four diarrheic field herds. The control litter was challenged with the homogenized intestinal tissue derived from one healthy herd.

Results

Diarrhea was reproduced in piglets from six litters challenged with intestinal tissue homogenate and these piglets developed intestinal pathology similar to that seen in the natural infection. Neither challenge with

the healthy herd homogenate nor with the filtrate of three case herd homogenates had any negative effect on the piglets.

Conclusions

Clinical signs and pathological changes seen in natural cases of New Neonatal Porcine Diarrhea Syndrome can be reproduced in newborn piglets by per oral inoculation with the intestinal tissue derived from naturally infected animals. We believe that this model will be valuable for studying the pathogenesis and development of the control, therapy strategies and eradication of this emerging syndrome in suckling piglets.

Keywords

Experimental infection; new neonatal porcine diarrhea

Background

Diarrhea in newborn piglets is a common problem in the swine production. It is often multifactorial and caused by an interaction between infectious agents, immunity of piglets and management procedures implemented on a farm. The pathogens commonly associated with neonatal porcine diarrhea are rotavirus A, coronavirus, enterotoxigenic *Escherichia coli*, *Clostridium perfringens* type A and C, *Clostridium difficile*, *Cryptosporidium* spp., *Giardia* spp., *Cystoisospora suis*, and *Strongyloides ransomi* [1-3]. However, in recent years, a new type of neonatal diarrhea in which none of the above mentioned pathogens can be identified as a causative agent, seem to have emerged in Denmark and Sweden [4-7]. In order to investigate this diarrheal syndrome, referred to as New Neonatal Porcine Diarrhea Syndrome (NNPDS), detailed epidemiological, pathological and microbiological studies have been conducted on four affected Danish pig farms [8-11]. Pathologically, the diarrheic piglets were dehydrated, had poor body condition and developed unspecific gross and histological lesions. The main pathological findings were flaccid intestines with fluid contents, villous atrophy and mild epithelial lesions. Diarrhea was neither related to insufficient colostrum intake nor infection with any of the common agents; however, identification of the exact etiology of this syndrome has so far been unsuccessful [8].

The purpose of the present study was to establish a reproducible animal model of NNPDS, which would provide basis for further investigations and establishment of prevention methods. We hypothesized the etiology of NNPDS to be of an infectious nature and that newborn piglets inoculated orally with intestinal tissue homogenate derived from naturally infected animals will develop clinical symptoms and pathological changes characteristic for this syndrome.

Methods

The experiment was approved by the Danish Animal Experiments Inspectorate.

Experimental animals, housing

In total 11 pregnant sows (Landrace x Yorkshire) were purchased at 4-6 weeks before farrowing from a commercial Danish high health herd. According to the farmer and veterinary practitioner's statement, this herd had not experienced problems with neonatal diarrhea of unknown etiology. The sows were housed in the facilities of the National Veterinary Institute, Technical University of Denmark, kept in individual stalls in separate and isolated pens with concrete floor and ad libitum access to water. The creep area in each pen was supplied with straw bedding and an overhead heat lamp. During the housing period the sows did not show signs of any disease. The piglets involved in the study were delivered naturally and no cross-fostering was made at any time of the experiments. Still-born piglets and piglets that died prior to inoculation were excluded from further examination. In total, 140 piglets (11 litters) were included in the investigation. No antibiotic therapy was given to the piglets at any time of the experiments.

Inocula

Source of inocula

Intestinal tissues for inoculation were obtained from the ileum and colon of 51 diarrheic piglets aged 3-7 days, collected from four Danish conventional pig herds during outbreaks of neonatal diarrhea, suspected

to represent NNPDs. The piglets were microbiologically tested for presence of *E. coli*, *C. perfringens* type A and C, *C. difficile* and rotavirus A. For further details see Kongsted et al., 2013 [8]. Four different inocula (inoculum 1-4) were prepared; each inoculum corresponding to one herd.

The source of the inoculum for the control litter (inoculum C) was the intestinal tissue from jejunum and colon derived from five healthy 4-day-old piglets collected from a commercial Danish herd that did not experience problems with neonatal diarrhea. These piglets were microbiologically examined for presence of enterotoxigenic *E. coli* and rotavirus A with negative results.

The source of the inoculum used for challenging the piglets from litter 8 was the intestinal tissue from jejunum and colon derived from the piglets from litter 2, previously challenged with one of the inoculum prepared from field cases.

Preparation of inocula

a. Homogenized intestinal tissue (inoculum C, inocula 1-4 and 1P)

The intestinal material was collected during necropsies, stored at -80°C and thawed before the trial. 30% inoculum was prepared by mixing 21g of intestinal tissue pooled from diarrheic piglets with 49 ml of sucrose-potassium-glutamate (SPG, pH 7.0) followed by homogenizing in a laboratory blender for 2 min. 10 % inoculum was prepared by dilution of 30% inoculum in SPG.

b. Homogenized intestinal tissue filtrate (inocula 1F, 2F and 3F)

The homogenized intestinal tissue filtrates was prepared from inocula 1-3. The inocula were filtrated through gauze, centrifuged at 10.000 g at 4°C for 20 min and passaged through series of filters with decreasing pore size: 1.45 µm, followed by 0.45 µm and 0.22 µm. The filtrates were then kept at 4°C until use. Absence of bacteria in the filtrated inocula was confirmed by aerobic and anaerobic cultivation on Columbia blood agar plates (Difco) supplemented with 5% bovine blood at 37°C for 3 days.

The inocula used in this study are listed in Table 1.

Study design

In total 11 experimental challenges were performed during a period of one year. The inoculation was done minimum six hours after the delivery, allowing the piglets uptake of colostrum prior to inoculation. The piglets were inoculated on the day of birth, day 1 or day 2 post-partum (PP). Inocula were administered orally using 5 mL sterile syringes.

In the first trial performed on litter 1, which served as a control litter, the piglets were randomly assigned to one of three groups (six piglets in each group) in order to determine the maximum concentration of inoculum tolerated by piglets. One group received 5 ml of 30% inoculum C; the second group received 5 ml of 10% inoculum C and the third group did not receive inoculum. None of the piglets in this litter developed diarrhea at any time of the experiment, thus 5 mL of 30% inoculum was used for challenging piglets in the other litters. However, the piglets in litters 10 and 11 were challenged with 3 ml, due to insufficient amounts of the inocula.

Depending on the litter size and available amount of the inoculum, seven to nine randomly selected piglets received the inoculum and the rest remained non-inoculated. Clinical examinations of sows and piglets were performed daily from day 1 to day 10 post inoculation (PI). In addition, rectal swabs were taken from each animal. General body condition and consistency of the feces on the rectal swab were recorded for each piglet. A piglet was considered to have diarrhea when the feces was liquid. Euthanasia and necropsies were scheduled for day 4, 6, and day 10 post-partum (PP), however the exact time point of euthanasia was determined by animal condition and technical staff availability. The selection of the piglets for euthanasia was random whenever animal welfare considerations did not speak against this procedure. Based on these considerations, the piglets showing poor body condition, dehydration or reduced viability, were selected for the necropsies prior to animals with good body condition. The sows were sacrificed on the termination of the experiment on day 9-12 PI. Data on the study design are presented in Table 2.

Necropsy and Histopathology

Gross observations were recorded for each piglet at necropsy. Tissue specimens for histopathological examination were collected from mid-jejunum, ileum and spiral colon. The specimens were fixed in 10 % neutral buffered formalin for minimum of 48 hours and were then embedded in paraffin blocks, cut at 3 µm and stained with hematoxylin and eosin (HE) following routine laboratory procedures. Light microscopic examination was carried out as previously described in Kongsted *et al.*, 2013 [8]. In short, three transverse sections of the jejunum, two transverse sections of the ileum and one transverse section of the colon from each piglet were evaluated blindly by the first author. The following features were assessed: the presence of villous atrophy and crypt hyperplasia, epithelial lesions, inflammation and necrotic changes in the intestinal mucosa, and the presence of intestinal parasites.

Microbiology

The microbiological tests were performed on the piglets from litters 2-11. The intestinal contents from the small intestines were examined for *E. coli* and *C. perfringens* by bacterial culturing and for rotavirus A by ELISA. The intestinal contents from the colon were anaerobically cultured for *C. perfringens* and *C. difficile*. Serogrouping of *E. coli* was performed by agglutination with monovalent O-antisera: O8, O45, O64, O138, O139, O141, O149 and O157 (State Serum Institute, Copenhagen, Denmark)[12] and typing of *C. perfringens* was done by conventional PCR and gel-electrophoresis [13].

Results

Clinical outcome

The control litter (litter 1) and the litters inoculated with homogenized intestinal tissue filtrates (litters 9-11)

No diarrhea was observed in these piglets at any time of the experiment. The piglets were normally hydrated and had normal body condition.

Litters challenged with intestinal tissue homogenate originating from diarrheic field cases (litters 2-8)

Diarrhea was observed in 52 piglets (75%) from litters 2, 3, 4, 5, 7, and 8 while the prevalence varied between 42 and 100% within the litter. Clinical symptoms were found in both inoculated and non-inoculated piglets between day 1 and day 7 PI (Figures 1-4). The clinical course of diarrhea was most severe

in litter 2 as compared to other litters. In litter 2 all piglets developed diarrhea with evident poor body condition and dehydration on day 2 and day 3 PI and were euthanized for animal welfare reasons on day 3 PI. The piglets from litters 3, 4, 5, 7, and 8 exhibited a milder diarrhea and in these litters the clinical condition of piglets was generally good. No clinical symptoms at any time of the experiment were observed in piglets from litter 6. In total, 37 piglets were submitted to euthanasia during a diarrheic stage (diarrhea present at euthanasia or one day prior to euthanasia). The remaining 15 diarrheic piglets were euthanized at least two days after the onset of diarrhea. Four piglets died during the experiment. One of these piglets (from litter 5) had diarrhea one day before death and the remaining piglets (one from litter 3 and two from litter 7) showed no diarrhea at any time.

Gross lesions

The control litter (litter 1) and the litters inoculated with homogenized intestinal tissue filtrates (litters 9-11)

Macroscopically, the piglets from the control litter (litter 1) and the litter inoculated with the filtrated inoculum 1 (litter 9) showed no pathological changes. In one piglet from litter 10 and two piglets from litter 11, a slight edema in mesocolon was the only lesion seen at necropsies.

Litters challenged with intestinal tissue homogenate originating from diarrheic field cases (litters 2-8)

In the diarrheic litters (litters 2-5, 7, and 8) watery contents in the small intestines and/or fluid-creamy contents in the large intestines were the main gross findings. This was seen in 35 piglets (51%) from these litters, both inoculated and non-inoculated, primarily in those that were submitted to euthanasia during the diarrheic stage. In 20 piglets (29%) from these litters a slight edema was seen in mesocolon. Additionally, in eight piglets (12%) from these litters the intestines were dilated and atonic. In litter 6, a slight edema in mesocolon was the only lesion observed at necropsies and it was seen in five piglets. In piglets that died during the experiment the following lesions were observed: an empty stomach in the piglet from litter 3 and subcutaneous hematoma in the piglet from litter 5. The remaining two dead piglets from litter 7 had no gross lesions.

Histopathology

In the piglets from the control litter (litter 1), the litter inoculated with inoculum 4 at day 2 PP (litter 6) and the litters inoculated with filtrated inocula 2 and 3 (litters 10 and 11) no histological lesions were seen in the intestinal tissue. In five piglets from the litter inoculated with the filtrated inoculum 1 (litter 9), a local, mild to moderate infiltration with neutrophils was seen in the lamina propria of the small intestinal mucosa.

In the piglets from the diarrheic litters (litters 2-5, 7, and 8) villous atrophy in the jejunum and/or ileum and mild lesions in the colonic epithelium were the most frequent findings. Villous atrophy was frequently associated with intestinal crypts elongation and was present in the intestines of 36 piglets (52%) from these litters (Figure 5). Epithelial lesions in colon including flattening, excessive shedding, small erosions and necrotic changes in the superficial enterocytes were seen in 13 piglets (19%) from these litters (Figure 6). Villous atrophy and epithelial lesions were typically seen in those piglets that were submitted to euthanasia during the diarrheic stage, regardless of whether they were inoculated or not.

Additional histological lesions were seen in the piglets from litter 2. These lesions included capillary congestion, hyperemia and edema in the mucosal lamina propria and occasionally in the submucosa. A number of bacteria were present in the intestinal lumen, between the villi and in close association with the villous epithelium (Figure 7).

Evaluation of the histological lesions in four piglets that died during the experiments was impossible due to tissue autolysis.

Clinical observations and the main gross and histological lesions are presented in Table 3.

Microbiology

In total 93% of the piglets from the litters challenged with inocula originated from field cases (litters 2-11) were positive for non-hemolytic *E. coli* in the aerobic culture. In 10% of the piglets (two piglets from litter 4, one from litter 5, eight from litter 7 and one from litter 11) *E. coli* O157 type was demonstrated. Additionally, 3% of the piglets (three from litter 10 and one from litter 11) were positive for *E. coli* O8. The majority of *E. coli* isolates were serologically non-typeable. In the anaerobic culture *C. perfringens* type A was found in 64% of the piglets from all litters regardless of the presence of diarrhea. Neither *C. perfringens* type C nor *C. difficile* were detected in any of the piglets. Rotavirus was detected in nine piglets (7%) among which eight were submitted to euthanasia during the diarrheic stage.

The summary results of the microbiological findings in both- experimentally and naturally infected piglets are shown in Table 4.

Discussion

This study was designed to support and continue previously undertaken investigations of NNPDS. The experiments showed that clinical signs and histological lesions similar to those seen in field cases of NNPDS can be found in 1-7-day-old piglets after per oral inoculation with 30% intestinal tissue homogenate from naturally infected animals. Moreover, diarrhea can be reproduced after one passage of the inoculum through an animal. Although the presently described model has its limitations, the results of this experiment indicate that NNPDS is of infectious nature.

Four inocula of different origins were used for inducing diarrhea in this investigation; each inoculum corresponded to one herd previously investigated in a field study [8]. As the exact causative agent of NNPDS has not yet been determined, we decided to prepare the inocula from the whole intestinal tissue of the field cases. Successful reproduction of infection using a tissue homogenate from naturally diseased animals has previously been described in relation to diseases without initially obvious etiology, e.g. runting and stunting syndrome in poultry [14, 15], porcine proliferative enteropathy [16, 17], and post-weaning multisystemic wasting syndrome [18].

Although there are some disadvantages of using tissue homogenate (i.e. uncontrolled infectious dose and impact of the tissue factors), this method is relatively easy to perform. Moreover, it has been shown that in some enteric infections inoculation with intestinal homogenate results in development of clinical signs and lesions similar to those reproduced by using a pure culture of a microorganism [19].

In this study diarrhea was successfully reproduced in 75% piglets from six litters inoculated with intestinal tissue homogenate. However, some inoculated piglets did not develop clinical symptoms. Similar observations were made in a study on mucohaemorrhagic diarrhea in pigs induced experimentally by using tissue homogenate and the author speculated that it could be due to biological variations between the animals, pre-existing immunity or inadequate infectious dose [20]. Similar speculations are relevant in this investigation. Furthermore, the clinical signs and histological lesions were also developed in some non-challenged piglets in this study. As the inoculated and non-inoculated piglets were kept together, the presence of diarrhea in non-inoculated animals resulted most likely from a transmission of the infection from inoculated piglets. However, it is also possible that an accidental self-inoculation by licking rests of the inoculum (e.g. from contaminated mammary gland skin) has occurred. In order to avoid misinterpretation of the results, an isolation of the inoculated animals from the non-inoculated ones should be implemented when using this model in future studies.

The course of experimentally induced diarrhea differed between the litters. The animals in litter 2 challenged with inoculum originating from herd 1 were most severely affected. Inoculation with the intestinal tissue from these piglets resulted in development of diarrhea with milder clinical course (litter 8). Whether this was due to the diminished virulence of the inoculum after one passage through an animal or differences in immune status between the litters remains unknown. Additionally, inoculum 4 used for challenging piglets in litters 6 and 7 seemed to be the least virulent resulting in a total absence of diarrhea (litter 6) or presence of mild diarrhea after a prolonged incubation period (litter 7). These observations correspond well with the clinical outcome of diarrhea in the field study reported by Kongsted *et al.*, 2014 [9, 10]. The piglets from Herd 1 were most severely affected by NNPDs in terms of the prevalence, timing and duration of diarrhea. The mortality as well as the prevalence of enteritis was also higher in these piglets compared to other herds [9]. These dissimilarities in the course of diarrhea between the herds could affect the virulence of inocula used in this study, resulting in diverse severity of the symptoms in the experimental animals.

Animal age at infection also seems to have affected the clinical outcome of diarrhea in this experiment. Two litters (litters 4 and 6) were inoculated at day 2 PP. In litter 4, 42% of the inoculated piglets developed diarrhea and in litter 6 none of the animals showed any symptoms. However, in repeated experiments with the same inocula administered at day 0 (litter 5) and 1 PP (litter 7), diarrhea occurred in 100% and 54% of the piglets, respectively. This suggests that the piglets are more susceptible to NNPDs when the introduction of the infection occurs during the first few hours of life.

The microbiological findings in the experimentally infected piglets were similar to those observed in the field study [8]. Non-hemolytic, in general non-typeable *E. coli* and *C. perfringens* type A were the main microbiological finding in the experimentally infected piglets. *C. perfringens* type A was found in 64% of the piglets in this investigation, both from diarrheic and non-diarrheic litters, thus its role as a causative agent of this diarrhea seems dubious. However, further studies on clostridial toxins are necessary in order to determine the importance of this bacterium in NNPDs. Neither hemolytic *E. coli*, *C. perfringens* type C, *C. difficile* nor parasites were detected in the piglets with experimentally induced NNPDs. Rotavirus A, considered to be an important pathogen in neonatal piglets, was only detected in nine experimentally challenged piglets and this relatively low prevalence suggests other etiologies underlying the investigated

diarrhea. Furthermore, no clinical signs could be reproduced in the piglets from the litters challenged with the inocula deprived of bacteria, which indicates a bacterial rather than a viral nature of NNPDS.

Gross and microscopic lesions observed in the experimental piglets with diarrhea resembled those seen in the naturally infected animals [8]. Similarly to the field cases, the diarrheic piglets with experimentally induced NNPDS had non-specific macroscopic changes, among which fluid intestinal contents were seen most frequently. However, the flaccidity of the intestines, which was the predominant finding in field cases, was only seen in 8 piglets in this investigation. This could be explained by the fact that the total number of experimentally infected piglets having diarrhea at the day of necropsy was relatively low and perhaps insufficient to determine whether flaccid intestines are characteristic for NNPDS under experimental conditions. Additionally, the subjective evaluation of this finding by the single person performing the necropsies could contribute to these results.

Histologically, villous atrophy with crypt hyperplasia was the predominant lesion in the piglets with experimentally induced NNPDS. This finding is similar to that reported in naturally infected animals and suggests that the presently investigated diarrhea results from decreased mucosal surface area available for absorption [21]. However, in similarity to the field study, the microbiological, clinical, gross and microscopic examination showed no evidence for pathogens commonly associated with villous atrophy such as rotavirus, coronavirus or intestinal parasites [22-25].

Four piglets died during this experiment. In two of them the post-mortem examination indicated starvation and trauma as possible causes of death and in the other two piglets the establishment of diagnosis was not possible. However, due to the lack of clinical symptoms in these piglets, it was concluded that the deaths were not related to NNPDS.

Conclusions

This study showed that NNPDS is of infectious nature and that clinical signs and histological lesions characteristic of this syndrome can be experimentally reproduced by per oral inoculation of newborn piglets with intestinal tissue derived from field cases and after one passage through an animal. We believe that this model will be of a great value in further studies on the pathogenesis and will enable development of the control, therapy strategies and eradication of this emerging syndrome in suckling piglets.

Animal ethics

The study was conducted with compliance with general ethical principles and informed client consent. All farms providing animals presented high standards of veterinary care.

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Competing interests

The authors of this research paper have no competing interests.

Author's contributions

All authors contributed to the design of this study. BJ, ØA and TKJ conducted animal experiment. Selection of the herds and animals that were source of inocula were performed by HK. All authors participated in drafting and proofreading of the manuscript. All authors approved the final version of the manuscript.

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Tables

Table 1. Inocula used in the study.

Litter	Inoculum	Source
1	C	Healthy piglets
2	1	Case animals from herd 1 ^a
3	2	Case animals from herd 2 ^a
4	3	Case animals from herd 3 ^a
5	3	Case animals from herd 3 ^a , repeated experiment
6	4	Case animals from herd 4 ^a
7	4	Case animals from herd 4 ^a , repeated experiment
8	1P	Piglets from litter 2
9	1F	Filtrated inoculum 1
10	2F	Filtrated inoculum 2
11	3F	Filtrated inoculum 3

^a: Origin of inoculum is herd 1-4 in Kongsted *et al.*, 2013

Table 2. Experimental study design.

Litter	Inoculum	Farrowing day relative to day of inoculation	Litter size	No. of piglets inoculated	No. of dead piglets	No. of piglets euthanized on day PI							
						2d	3d	4d	5d	6d	7d	8d	9-12d
1	C	-1	18	12			6				6		6
2	1	-1	13	9			13						
3	2	0	16	9	1		4			7			4
4	3	-2	12	9		4		4				4	
5	3	0	7	7	1			2		2	1		1
6	4	-2	12	9		4		4				4	
7	4	-1	13	9	2			4			4		3
8	1P	0	8	8				3	3				2
9	1F	-1	12	9			4			4			4
10	2F	0	15	7				4	3	5			3
11	3F	0	14	7				4	3	4			3

Table 3. Clinical observations and main gross and histological lesions in experimental piglets.

	1	2	3	4	5	6	7	8	9	10	11
Litter	n=18	n=13	n=16	n=12	n=7	n=12	n=13	n=8	n=12	n=15	n=14
Clinical signs											
Diarrhea, total (%)	0	100	81	42	100	0	54	88	0	0	0
Diarrhea, 2 or more days (%)	0	92	69	25	86	0	31	62	0	0	0
Diarrhea, total (no.)	0	13	13	5	7	0	7	7	0	0	0
Inoculated	0	9	8	5	7	0	4	7	0	0	0
Non-inoculated	0	4	5	0	NA	0	3	NA	0	0	0
Poor body condition	0	13	3	1	2	0	0	2	0	0	0
Dehydration	0	13	3	0	1	0	0	0	0	0	0
Gross lesions											
Dilated, atonic intestines	0	0	5	3	0	0	0	0	0	0	0
Watery content in small intestine	0	13	7	3	3	0	0	3	0	0	0
Fluid-creamy content in large intestine	0	13	8	4	4	0	3	3	0	0	0
Edema in mesocolon	0	1	1	5	3	5	3	2	0	1	2
Histological lesions											
Villus atrophy with crypt hyperplasia	0	13	7	5	5	0	4	2	0	0	0
Epithelial lesions in colon	0	0	4	4	2	0	0	2	0	0	0

Table 4. Microbiological findings in piglets naturally infected with NNPDS (**Herd 1-4**)¹ vs. piglets with experimentally induced NNPDS (litter 2-11).

	Non-hemolytic <i>E. coli</i>	Hemolytic <i>E. coli</i>	<i>C. perfringens</i> type A	<i>C. perfringens</i> type C	<i>C. difficile</i>	rotavirus A
Herd 1 (n=13)	8	3	3	2	0	0
litter 2 (n=13)	13	0	2	0	0	0
litter 8 (n=8)	8	0	1	0	0	0
litter 9*(n=12)	12	0	11	0	0	0
Herd 2 (n=11)	10	0	2	0	0	1
litter 3 (n=15 ^a)	15	0	6	0	0	5
litter 10*(n=15)	7	0	12	0	0	0
Herd 3 (n=14)	5	0	4	0	0	0
litter 4 (n=12)	12	0	10	0	0	0
litter 5 (n=7)	7	0	2	0	0	1
litter 11*(n=14)	14	0	13	0	0	0
Herd 4 (n=13)	11	0	9	1	0	0
litter 6 (n=12)	12	0	11	0	0	0
litter 7 (n=13)	13	0	10	0	0	3

¹ Herd 1-4 in Kongsted *et al.*, 2013^a One piglet excluded from microbiological testing

*Challenged with filtrated inoculum

Figures

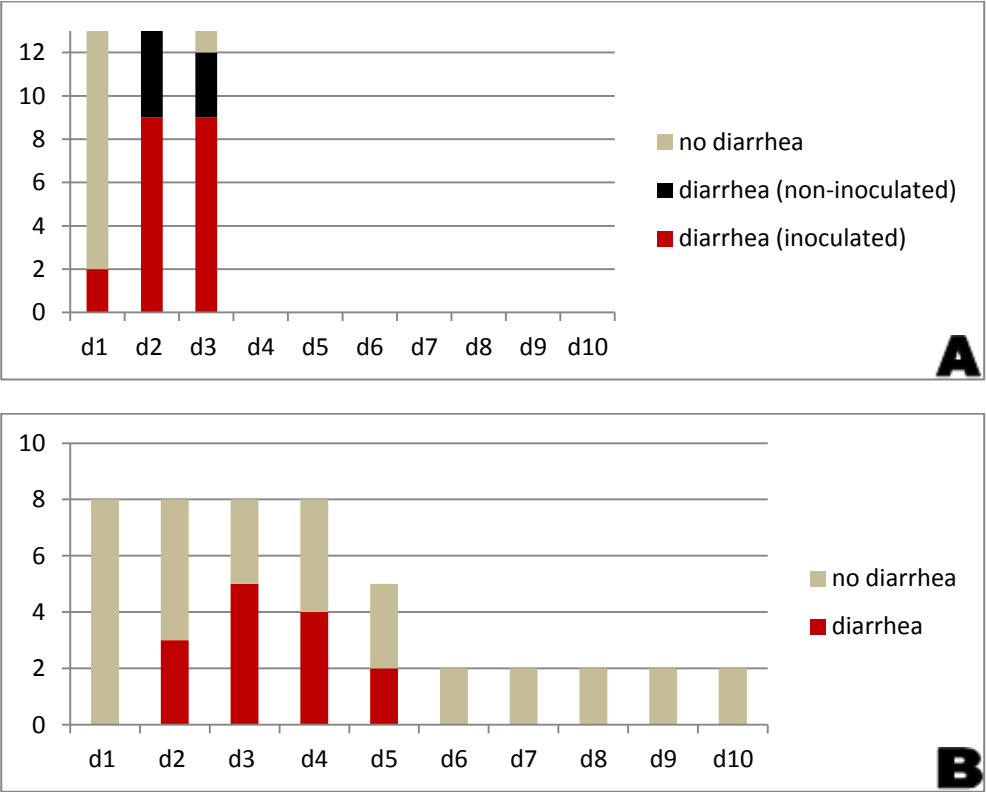


Figure 1. Clinical course of infection in litters challenged with inoculum originated from herd 1 (x- day PI, y- no. of piglets).
A. Litter 2 challenged with intestinal tissue homogenate from field cases.
B. Litter 8 challenged with intestinal tissue homogenate from piglets from litter 2 (passed inoculum).

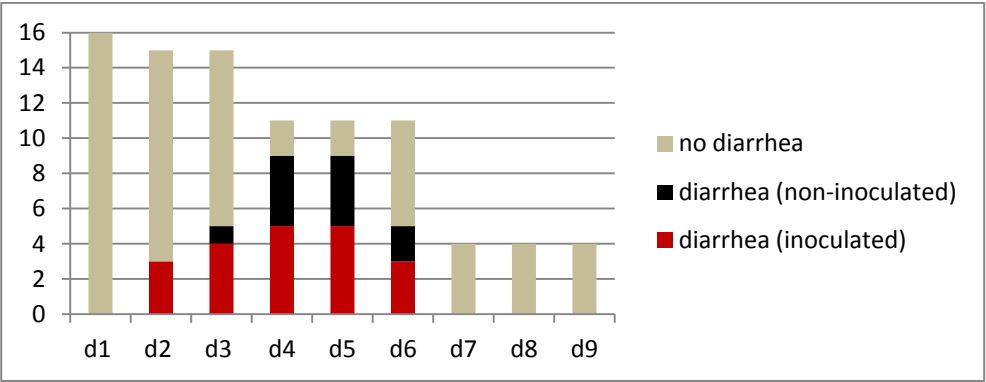


Figure 2. Clinical course of infection in litter 3 challenged with inoculum originated from herd 2 (x- day PI, y- no. of piglets).

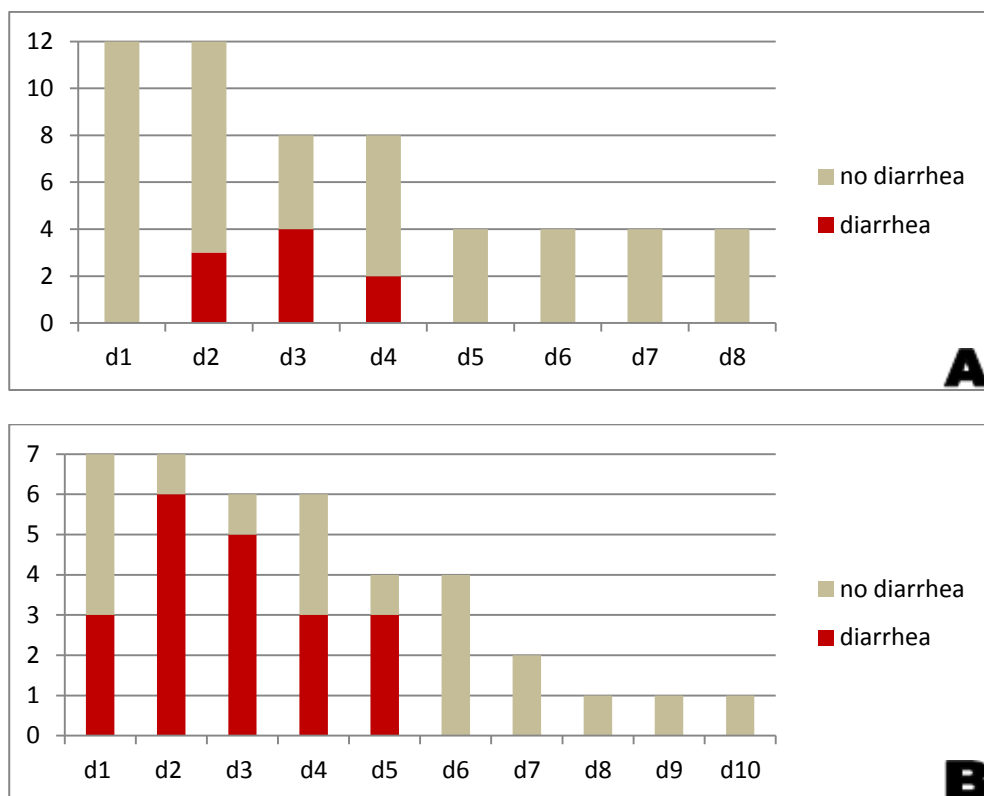


Figure 3. Clinical course of infection in litters challenged with inoculum originated from herd 3 (x- day PI, y- no. of piglets).

A. Litter 4 challenged at day 2 PP.

B. Litter 5 challenged at the day of birth.

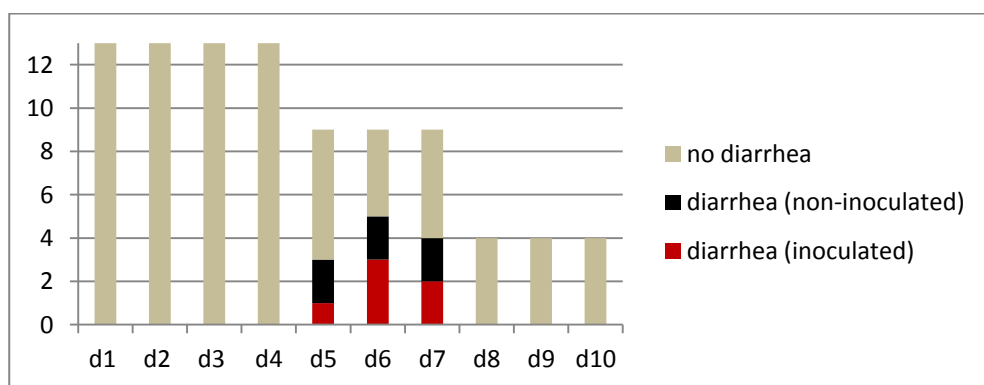


Figure 4. Clinical course of infection in litter 7 challenged with inoculum originated from herd 4 at day 1 PP (x- day PI, y- no. of piglets).

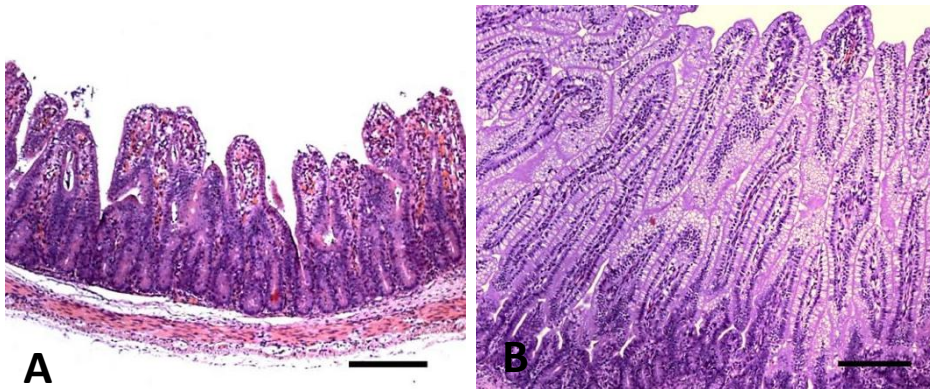


Figure 5. Sections of the jejunum from experimental piglets.

A. Villus atrophy with crypt hyperplasia in a diarrheic piglet challenged with a case herd homogenate, HE, bar 200 μm .

B. Normal intestinal mucosa from a control piglet challenged with a healthy herd homogenate, HE, bar 200 μm .

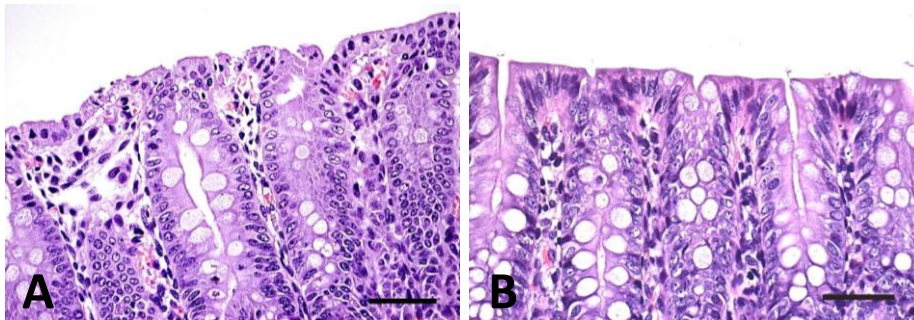


Figure 6. Sections of the colon from experimental piglets.

A. Flattening and necrosis of the luminal enterocytes in the colon of a diarrheic piglet challenged with a case herd homogenate, HE, bar 50 μm .

B. Normal colonic epithelium in a control piglet challenged with a healthy herd homogenate, HE, bar 50 μm .

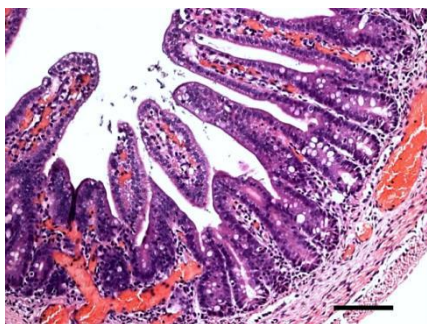


Figure 7. Histological lesions in the jejunum of a piglet from litter 2; villus atrophy with crypt hyperplasia, hyperemia in the lamina propria and capillary congestion, HE, bar 100 μm .

6. Accompanying paper

Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome

Hanne Kongsted, Beata Jonach, Svend Haugegaard, Øystein Angen, Sven E Jorsal, Branko Kokotovic, Lars E Larsen, Tim K Jensen and Jens P Nielsen

RESEARCH ARTICLE

Open Access

Microbiological, pathological and histological findings in four Danish pig herds affected by a new neonatal diarrhoea syndrome

Hanne Kongsted^{1,3*}, Beata Jonach², Svend Haugegaard¹, Øystein Angen², Sven E Jorsal², Branko Kokotovic², Lars E Larsen², Tim K Jensen² and Jens P Nielsen³

Abstract

Background: Neonatal diarrhoea is a frequent clinical condition in commercial swine herds, previously regarded to be uncomplicated to treat. However, since 2008 it seems that a new neonatal diarrhoeic syndrome unresponsive to antibiotics and common management practices has emerged. Routine laboratory examinations have not detected any pathogen related to this syndrome. The primary purpose of this study was to evaluate if well-known enteric pathogens could be associated with outbreaks of neonatal diarrhoea, thus question the hypotheses of a new syndrome. Furthermore, we wanted to evaluate macroscopic and microscopic findings associated with these outbreaks and if possible propose a preliminary piglet-level case-definition on syndrome New Neonatal Porcine Diarrhoea syndrome (NNPDS).

Results: Four well-managed herds experiencing neonatal diarrhoea with no previously established laboratory conclusion and suspected to suffer from New Neonatal Porcine Diarrhoea Syndrome, were selected. Within these herds, 51 diarrhoeic and 50 non-diarrhoeic piglets at the age of three to seven days were necropsied and subjected to histological and microbiological examination. Faeces were non-haemorrhagic. Neither enterotoxigenic *E. coli*, *Clostridium perfringens* type A or C, *Clostridium difficile*, rotavirus, coronavirus, *Cryptosporidium* spp, *Giardia* spp, *Cystoisospora suis* nor *Strongyloides ransomi* were associated with diarrhoea in the investigated outbreaks. Macroscopically, the diarrhoeic piglets were characterized by filled stomachs and flaccid intestines without mucosal changes. The predominant histological lesions were villous atrophy in jejunum and ileum. Epithelial lesions in colon were seen in one third of the case piglets.

Conclusions: The results of the study supported the hypothesis that a new neonatal porcine diarrhoea was present in the investigated herds, since no known pathogen(s) or management factors could explain the diarrhoeal outbreaks. Based on the findings in the four herds the following case-definition of NNPDS was suggested: Non-haemorrhagic diarrhoea during the first week of life, without detection of known infectious pathogens, characterized by milk-filled stomachs and flaccid intestines at necropsy.

Background

Neonatal diarrhoea is a well-known clinical condition, present at varying prevalence in most commercial swine herds. However, since 2008 field experiences on an apparently new diarrhoeic syndrome unresponsive to antibiotics and common management practices have been

reported (personal communications, S.E. Jorsal, National Veterinary Institute, Technical University of Denmark and B. Svensmark, Pig Research Centre, Danish Agriculture & Food Council, Denmark). The emergence of a new neonatal diarrhoeic syndrome (by some authors referred to as New Neonatal Porcine Diarrhoea (NNPD)) has been suggested in different countries [1-4]. A common feature of the reported cases is that known enteric pathogens cannot be associated with the clinical outbreaks in routine laboratory submissions.

Routine laboratory testing protocols may vary from region to region. Disregarding local procedures, the following

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agents are usually included in diagnostic protocols for neonatal diarrhoea: Enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens* type A (CPA), *Clostridium perfringens* type C (CPC), *Clostridium difficile* (CD) rotavirus group A (RV) and coronavirus [1,5]. Parasites, which may be relevant to consider in relation to neonatal diarrhoea are *Cryptosporidium spp*, *Giardia spp*, *Cystoisospora suis* and *Strongyloides ransomi* [6-8]. Systematic investigations of piglets from herds affected by the apparently new diarrhoeic syndrome are lacking.

The overall aim of this study was to investigate whether a detailed microbiological examination of a larger number of piglets from affected herds could link the presence of neonatal diarrhoea with known enteric pathogens. Such associations would challenge the hypothesis that a new disease syndrome has evolved. Another aim was to determine if diarrhoeic piglets from different herds had characteristic and consistent gross and microscopic lesions to support the elaboration of a joint case definition of NNPDs.

The article describes the prevalence of well-known enteric pathogens in age-matched diarrhoeic- and non-diarrhoeic piglets from four herds affected by neonatal diarrhoea with no previously established laboratory conclusion. Furthermore, results of gross pathology and histopathology are presented. Summarizing these findings, the article suggests a case-definition on NNPDs.

Results

Epidemiologic data on piglets

A total of 51 diarrhoeic (11–14 pr. herd) and 50 non-diarrhoeic piglets (12–13 pr. herd) at the age of three to seven days were included in the study. Clinically, the diarrhoeas were non-haemorrhagic. Eighty percent of diarrhoeic piglets had been diarrhoeic for either two or three days prior to euthanasia. Diarrhoea for four days was seen in 14% of the diarrhoeic piglets whereas only 6% had been diarrhoeic for five days.

Microbiology

Table 1 summarizes the microbiological findings in relation to diarrhoeic status. None of the microbiological agents was significantly more prevalent in diarrhoeic than in non-diarrhoeic piglets.

Non-haemolytic *E. coli* was the predominant finding in the aerobic culture from both diarrhoeic and non-diarrhoeic piglets, whereas haemolytic strains were found in only three piglets in total (all of them diarrhoeic). The main part of *E. coli* isolates were non-typeable. Sixty-three *E. coli* isolates were subjected to virulence gene determination by PCR. Fimbrial genes were detected in nine of 35 isolates from diarrhoeic piglets. The fimbrial distribution among isolates was; F4 (n=2), F5 (n=1), F6 (n=1), F18 (n=1), F41 (n=2), F5/F6 (n=1) and F5/F41 (n=1). In non-diarrhoeic piglets fimbrial genes were detected in seven of

Table 1 Microbiological findings in diarrhoeic and non-diarrhoeic piglets

Microbiological agent	Diarrhoeic n=51 (%)	Non-diarrhoeic n=50 (%)	P-value ¹
Haemolytic <i>E. coli</i> non-typable	6	0	0.1
Non-haemolytic <i>E. coli</i> non-typable	47	48	0.5
Non-haemolytic <i>E. coli</i> O-rough	4	2	0.5
Non-haemolytic <i>E. coli</i> serogroup O8	4	6	0.8
Non-haemolytic <i>E. coli</i> O157	8	0	0.06
<i>C. perfringens</i> type A	35	70	0.9
<i>C. perfringens</i> type C	6	2	0.3
<i>C. difficile</i>	0	4	0.5
rotavirus group A	2	0	0.5
coronavirus	0	0	1

¹: Positive associations with diarrhoea tested by One-sided Fisher's exact test.

28 isolates. The fimbrial distribution among these isolates was; F4 (n=1), F5 (n=1), F6 (n=1), F18 (n=2) and F41 (n=2). Table 2 gives an overview of toxin genes detected in fimbriated and non-fimbriated isolates from the two groups of piglets. Classic ETEC with simultaneous occurrence of both fimbrial and toxin genes were detected in only one diarrhoeic piglet.

In the anaerobic culture, CPA was a very frequent finding. These bacteria were more prevalent in non-diarrhoeic than in diarrhoeic piglets (70% vs. 35%).

Necropsy

Necropsy findings are presented in Table 3. Very few extra-intestinal lesions were observed (not shown), and only one of these; a pale or icteric liver, was observed in more than one piglet (5 of the diarrhoeic vs. 1 of the non-diarrhoeic piglets).

As indicated in Table 3, six findings showed a statistically significant higher prevalence in diarrhoeic versus non-diarrhoeic piglets. Table 4 outlines the prevalence of these six findings in the two groups of piglets within each herd. Within all herds, a poor body condition, flaccidity of the small intestine, flaccidity of the large intestine and liquid large intestinal contents seemed positively associated with diarrhoea (though not statistically significant in all cases, see Table 4). Flaccidity of the large intestine was in most cases (25 of 27 cases) seen in conjunction with small intestinal flaccidity. Figure 1 shows a flaccid and Figure 2 shows a normal intestine.

Histopathology

In the small intestine, villous atrophy with crypt hyperplasia was the most frequently observed lesion. Figure 3

Table 2 Occurrence of toxin genes within fimbriated and non-fimbriated *E. coli* isolates from diarrhoeic and non-diarrhoeic piglets

<i>E. coli</i> isolates	n	STa ¹	STb ¹	LT ²	VT2e ³	STa/LT	STb/VT2e	LT/VT2e
From diarrhoeic piglets								
Fimbriated	9	1	0	0	0	0	0	0
Non-fimbriated	26	1	2	2	5	0	0	0
From non-diarrhoeic piglets								
Fimbriated	7	0	0	0	3	0	0	0
Non-fimbriated	21	1	0	0	3	1	1	1

¹: Heat-stable enterotoxins a and b. ²: Heat-labile enterotoxin. ³: Verotoxin 2e.

shows atrophic villi in ileum as compared to normal villi shown in Figure 4. Overall, an atrophic pattern was seen in the jejunal and/or ileal mucosa in 63% of diarrhoeic and 12% of non-diarrhoeic piglets. The severity of atrophy varied, with no obvious association with diarrhoeic status. In ileum, the villous atrophy was most pronounced over the Peyer's patches. Duodenal villi were not affected. A statistically significant association ($P < 0.001$) between villous atrophy and flaccidity of the small intestine at necropsy was seen. In 76% of piglets having villous atrophy, small intestinal flaccidity had been recorded at necropsy.

Irrespective of diarrhoeic status approximately 30% of piglets had a slight to moderate local infiltration of

neutrophils in the lamina propria. Occasionally, the lamina propria in the diarrhoeic piglets was congested and edematous.

Mild epithelial lesions were seen at the tip of the villi in 20% of the diarrhoeic and 6% of the non-diarrhoeic piglets and were usually associated with villous atrophy. Crypts of Lieberkühn epithelium were intact in both groups. Foci of mucosal necrosis were seen in the small intestines of 6% of the diarrhoeic piglets versus none of the non-diarrhoeic ones. In colon, mild epithelial lesions were seen in 33% of the diarrhoeic piglets and 11% of the non-diarrhoeic piglets. Occasionally, the colonic crypts in diarrhoeic piglets were irregular and elongated. Mucosal necrosis in the colon was seen in one diarrhoeic piglet, which also had necrotic changes in the small intestine.

No parasites were seen in the intestinal mucosa of any piglet.

Table 5 depicts histopathological findings in diarrhoeic and non-diarrhoeic piglets within the four herds and summarizes the overall prevalences. Both villous atrophy and large intestinal epithelial lesions showed an overall statistically significant positive association with diarrhoea, and seemed positively (or at least not negatively) associated with diarrhoea within all herds.

Discussion

Non-enterotoxigenic (containing neither fimbrial nor toxin genes) *E. coli* was a frequent finding in both diarrhoeic and non-diarrhoeic piglets, whereas only one enterotoxigenic isolate was detected. Hence, ETEC did not seem to play any pathogenic role in relation to the investigated outbreaks of diarrhoea. Other studies have indicated that attaching and effacing *E. coli* (AEEC), carrying neither fimbrial nor toxin genes, are able to induce diarrhoea in newborn piglets [9] and to induce villous atrophy [10]. The prevalence of AEEC in the present study is currently being investigated.

CPC was cultured in four piglets of the study. Due to the low prevalence the significance of this bacterium in relation to the investigated outbreaks is probably minimal. The significance of CPA in relation to diarrhoea in

Table 3 Necropsy findings in diarrhoeic and non-diarrhoeic piglets

Necropsy findings	Diarrhoeic n=51 (%)	Non-diarrhoeic n=50 (%)	P-value ¹
<i>General findings</i>			
Poor body condition	57	4	< 0.0001
Dehydration	29	2	< 0.0001
Empty stomach	0	12	1
<i>Small intestine</i>			
Flaccidity	73	20	< 0.0001
Hyperaemia of serosa	6	0	0.2
Stripping of serosa	2	0	1
Edema in mesentery	4	4	1
Enlargement of lymph nodes	18	16	0.9
Dullness/ necrosis of mucosa	8	8	1
Watery contents	57	30	0.01
<i>Large intestine</i>			
Flaccidity	53	6	< 0.0001
Edema in mesentery	39	20	0.06
Enlargement of lymphnodes	4	2	1
Liquid contents	48	10	< 0.0001

¹: One-sided Fisher's exact test. Findings having a statistically significant positive association with diarrhoea are presented in bold.

Table 4 Necropsy findings in diarrhoeic- (D) and non-diarrhoeic (ND) piglets in individual herds

Necropsy findings	Herd 1		Herd 2		Herd 3		Herd 4	
	D	ND	D	ND	D	ND	D	ND
	n=13 (%)	n=13 (%)	n=11 (%)	n=12 (%)	n=14 (%)	n=13 (%)	n=13 (%)	n=12 (%)
<i>General findings</i>								
Poor body condition	77*	15	63*	0	57*	0	31*	0
Dehydration	31*	0	0	0	57*	8	23	0
<i>Small intestine</i>								
Flaccidity	69*	15	81*	25	86*	0	54	42
Watery contents	92*	23	64*	17	36	31	38	50
<i>Large intestine</i>								
Flaccidity	62*	8	55*	8	64*	0	31	8
Liquid contents	46*	8	45	17	50*	8	46*	8

* denotes significant statistical positive association with diarrhoea (one-sided Fisher's exact test ($\alpha=0.05$)) within the individual herds.

neonatal piglets is controversial, since it has been concomitantly recognized as part of the normal intestinal flora and as a potential intestinal pathogen [11,12]. In this study we found a significantly higher prevalence of CPA in non-diarrhoeic vs. diarrhoeic piglets. Most likely, this finding merely reflects the intact intestinal flora within the non-diarrhoeic piglets. CD has been reported in cases of neonatal diarrhoea in piglets [5,12]. However, in this study, this bacterium was only detected in two piglets and the characteristic histopathological lesions previously reported to be associated with CD infections

[13] were not seen. Accordingly, CD does not seem to be associated with the investigated outbreaks.

The scarcity of known pathogens in the outbreaks of the study supports the hypothesis that the investigated herds experienced diarrhoea of unknown aetiology. Therefore the outbreaks may be representative of the new syndrome NNPDS.

A poor body condition and dehydration were rather prevalent findings in diarrhoeic piglets in this study. However, unless very pronounced, these features are not characteristic for specific diarrhoeic syndromes, since they merely reflect the loss of nutrients and water associated



Figure 1 Flaccid intestine of 3 days old diarrhoeic piglet. The small intestine is thin-walled and flaccid throughout its length. The intestine appears to lack its normal peristaltic capacity, since no sections are contracted. Colon (in the right side of the picture) has liquid contents.



Figure 2 Normal intestine of 3 days old non-diarrhoeic piglet. The small intestinal wall has a normal thickness. Different parts of the intestine show different stages of peristalsis, reflecting normal peristaltic capacity.

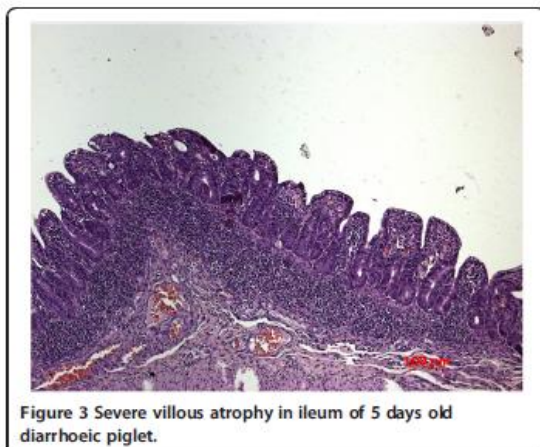


Figure 3 Severe villous atrophy in ileum of 5 days old diarrhoeic piglet.

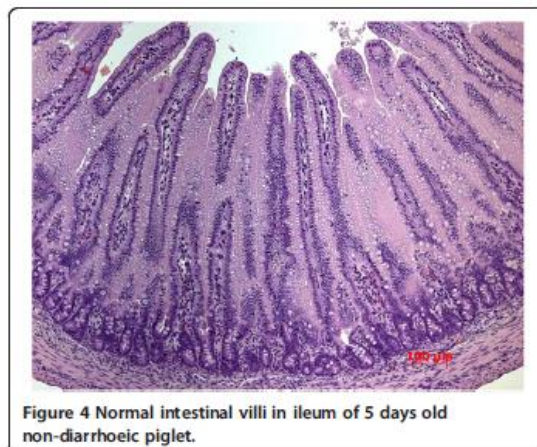


Figure 4 Normal intestinal villi in ileum of 5 days old non-diarrhoeic piglet.

with any diarrhoeic condition. Milk-filled stomachs, in contrast, seem to be a characteristic finding associated with this syndrome. Since neonatal diarrhoea is commonly associated with malabsorption caused by starvation, the filled stomachs seen in 100% of diarrhoeic piglets in this study are interesting findings which clearly differentiate this syndrome from outbreaks of neonatal diarrhoea related to starvation. However, since the vast majority (80%) of piglets in this study were diarrhoeic for two or three days only, we do not have information on the contents of stomachs at later stages of disease. Obviously, one would expect long lasting diarrhoea to keep piglets from suckling due to malaise, and therefore this criterion is probably only valid at early stages of disease.

Intestinal flaccidity was the most prominent and consistent gross lesion. Flaccidity of intestines is seen in different conditions, Postweaning Multisystemic Wasting Syndrome (PMWS) [14] and diet-induced malabsorption being the most obvious examples. Liquid contents in colon are expected in all diarrhoeic conditions and are therefore not considered diagnostic to any specific syndrome. Somewhat surprising, half of the diarrhoeic piglets did not have liquid content in colon at necropsy, which probably reflects that these piglets were in the recovery phase of disease. If so, this potentially implies a diagnostic problem due to less pronounced lesions and decreased excretion of infectious agents at this phase. However, since the clinical course of diarrhoea turned out to be very short (as evidenced by 80% of the selected piglets being diarrhoeic for only two or three days) it would not have been practically feasible to avoid selection of piglets in recovery.

The most consistent and predominant histological lesion observed in diarrhoeic piglets was villous atrophy (seen in 63% of diarrhoeic vs. 12% of non-diarrhoeal piglets). Villous atrophy is a very common finding in diarrhoeic conditions [15] and in this study, the atrophy was neither

associated with infection by well-known pathogens nor malnutrition. The strong association between villous atrophy and grossly visible intestinal flaccidity indicates that decreased mucosal thickness is reflected grossly as a thin-walled, atonic intestine. Epithelial lesions in the large intestine also seemed to be consistently associated with diarrhoea in this study (seen in 33% of diarrhoeic vs. 11% of non-diarrhoeic piglets), but due to the low prevalence, these lesions do not seem to be relevant to include in a case definition.

Overall, the present study suffers from lack of comparable piglets from non-NNPDS-affected herds in order to correctly classify findings as typical or diagnostic of NNPDS. Moreover, the selection of study herds posed some difficulties since the selection was basically based on a high prevalence of diarrhoea and absence of agents (in five piglets). Obviously, potential misclassification of herds is an issue to consider – though hard to address or control at this stage of investigation.

To our knowledge, this is the first study investigating outbreaks of diarrhoea in herds suspected to suffer from NNPDS. The aetiology behind these outbreaks was either undetected pathogens or non-infectious factors. Practical experience indicates that eg. high levels of protein in sow feed can lead to diarrhoea in neonatal pigs. However, all of the investigated herds used restricted levels of protein in sow feed, and had previously tried minimizing protein content with no preventive effect. As previously underlined, the diarrhoea seemed unrelated to postnatal starvation, but intrauterine events may have affected the normal development of intestinal absorptive capacity. Thus, the villous atrophy seen in the study may reflect prenatal under-development of villi.

The unspecific nature of intestinal lesions seen in this study underlines the complexity of intestinal pathology in neonatal pigs. Interestingly, even early studies from

Table 5 Main histological findings in 51 diarrhoeic (D) and 50 non-diarrhoeic (ND) piglets

Histological findings	Herd 1		Herd 2		Herd 3		Herd 4		In total	
	D	ND	D	ND	D	ND	D	ND	D	ND
	n=13 (%)	n=13 (%)	n=11 (%)	n=12 (%)	n=14 (%)	n=13 (%)	n=13 (%)	n=12 (%)	n=51 (%)	n=50 (%)
<i>Small intestine</i>										
Villous atrophy	62*	8	82*	8	71*	0	38	33	63	12
Neutrophil infiltration	38	38	55	25	21	15	23	50	33	32
Epithelial lesions	15	15	36*	0	21	0	8	8	20	6
Mucosal necrosis	8	0	0	0	0	0	15	0	6	0
<i>Large intestine¹</i>										
Epithelial lesions	17	15	55*	8	46	22	15	0	33	11
Mucosal necrosis	0	0	0	0	0	0	8	0	2	0

¹: In 2 diarrhoeic and 4 non-diarrhoeic piglets samples from colon were missing or autolytic and therefore not included in the analysis.

*denotes statistically significant positive associations with diarrhoea within herds (one-sided Fisher's exact test ($\alpha=0.05$)). Findings having a statistically significant association with diarrhoea across herds are presented in bold.

the seventies and eighties concluded that gross lesions seem similar and unspecific irrespective of the underlying aetiology in this age group of piglets [16,17]. Moreover, in the study from 1975 no pathogens were detected in as many as 32% of fatal neonatal gastroenteropathies. Thus, the existence of neonatal diarrhoea with unspecific lesions and without known pathogens is not a new phenomenon. It appears, however, that the clinical picture in the herds is new. At this point we do not know whether this clinical picture is a more severe manifestation of a syndrome already present but not recognized back in the seventies, or if we are experiencing a truly new syndrome.

From a practical point of view, obviously the most urgent issue is to recognize the aetiology behind the current problems. This study disclaims associations with established agents, but yet unestablished agents or shifts in intestinal bacterial population dynamics may play a role. Therefore, culture-independent methods like metagenomics and high-throughput qPCR may be rewarding in the future investigation.

Conclusions

This study suggests the existence of a yet unexplained diarrhoea syndrome related to the first week of life. The syndrome is not related to starvation or infection by enterotoxigenic *E. coli*, *Clostridium perfringens* type A or C, *Clostridium difficile*, rotavirus, coronavirus, *Cryptosporidium* spp, *Giardia* spp, *Cystoisospora suis* or *Strongyloides ransomi*. Characteristic postmortem findings are flaccid intestines without mucosal pathology or lymph node enlargement. Histologically, villous atrophy in jejunum and ileum are the most prominent findings and epithelial lesions in colon also seem to be associated with the syndrome. Apparently, the flaccidity of intestines seen at necropsy is a reflection of the reduced length of intestinal

villi. For a preliminary piglet level case-definition we suggest the following: Non-haemorrhagic diarrhoea during the first week of life, with no detection of known infectious agents and characterized by a milk-filled stomach and flaccid intestines at necropsy. Since histopathological examination mainly revealed uncharacteristic lesions, this diagnostic approach does not seem to be rewarding at this stage of investigation.

Methods

Study design

A case-control study on 101 euthanized piglets selected from four Danish production herds was performed during 2011. A total of 989 piglets from these herds were clinically evaluated from the day of birth and 110 were euthanized at selected time points.

Inclusion of herds

Herds were recommended by veterinary practitioners and included in accordance with the following criteria: 1) Presence of diarrhoea responding poorly to antibiotics during the first week of life (at least 30% affected litters for a period of minimum 6 months), 2) Routine vaccination of sows against ETEC and CPC, 3) Failure of preventive management interventions, 4) PRRS negative farrowing unit as demonstrated in blood samples tested by ELISA/IPT or PCR and 5) Negative results of routine diagnostic examinations for ETEC, CPC and RV in five diarrhoeic piglets aged one to four days.

A total of four herds were selected. They all presented high standards of housing and management with all-in/all-out practice in farrowing units and appropriate cleaning between farrowing batches. Farrowing crates had partially slatted floors of plastic or iron bars with supplemental heat and cover provided for the piglets.

All four herds had been affected by neonatal diarrhoea for at least one year. Preventive interventions which had failed included minimizing protein-levels in sow feed, optimization of hygiene procedures, immunization by faecal backfeeding and vaccination against CPA and Porcine circovirus type 2 (PCV2). In all herds, many different antibiotics and different treatment strategies had been tried out unsuccessfully. All herds used Toltrazuril at day three to four of life to prevent coccidiosis. Castration of males and iron-injections were carried out at the same day. Descriptive data on the herds are presented in Table 6.

Inclusion of sows and piglets

In each herd, approximately 20 newly farrowed sows (half of a farrowing batch) with no clinical signs of disease prior to farrowing were selected. The selection procedure was designed to include all available first parity litters, since they were expected to exhibit the highest prevalence of diarrhoea. At the day of birth (day one), the included litters were standardized to 11 or 12 piglets by randomly selecting piglets weighing ≥ 800 g. Surplus piglets were removed during the first 16 hours after birth and no cross-fostering was made during the suckling period. Sows and piglets were clinically examined daily from day 1 until day five to seven and again on day ten. In the same period, rectal swabs were taken. Consistency of faeces was judged as fluid or normal from the appearance on the rectal swab.

Definition on cases and controls and selection procedure

In each herd, age-matched case and control piglets were selected for necropsy at two different time-points – early and late in the course of disease. These time-points were based on previous experience with the syndrome in each herd. In herds experiencing diarrhoea starting at the second day of life, piglets were necropsied at day three and five of life. If diarrhoea occurred from the third day of life, piglets were necropsied at day four and six of life and so on. In the 4 herds, clinical signs started at day two, three or

four of life, resulting in necropsies being performed on piglets between three and seven days of age.

Selection criterion for diarrhoeic piglets was fluid consistency of faeces for at least two subsequent days, including the day of selection. Selection criterion for non-diarrhoeic piglets was normal consistency of faeces at all days prior to selection. Diarrhoeic piglets were selected from the litters having the highest prevalence of diarrhoea, whereas non-diarrhoeic piglets were selected from the litters exhibiting no or little diarrhoea. None of the piglets euthanized at the early stage (three to five days of age, depending on herd) had been treated by antibiotics. All diarrhoeic piglets euthanized at the late stage (five to seven days of age, depending on herd) had been medicated according to the individual herd routine.

Diagnostic procedures

Necropsy

Live piglets were transported to the laboratory and euthanized within six hours after selection. All organs were routinely examined for gross lesions. A poor body condition was recorded if protruding ribs and spine were observed. Dehydration was recorded if eye balls were deeply positioned in the skull and muscles appeared dry on the cut surface.

Histopathology

Histopathological examination was carried out on samples from duodenum, jejunum, ileum and spiral colon. Samples were fixed immediately after euthanasia in 10% neutral buffered formalin for at least 48 hours. The samples were then embedded in paraffin wax, cut at 3 μ m, stained with haematoxylin and eosin (HE) and examined by light microscopy. The intestinal mucosa of each specimen was histopathologically evaluated.

Villous atrophy was recorded when shortening of villi accompanied by decreased height of enterocytes and increased cellularity of the lamina propria was seen in at least one region of the intestinal sample. Crypt hyperplasia was recorded when the intestinal crypts were

Table 6 Descriptive data of the 4 herds in the study

Herd data	Herd 1	Herd 2	Herd 3	Herd 4
Study period	January 2011	March 2011	May 2011	July 2011
Herd size (number of sows)	900	1250	700	950
SPF ¹ -status	Not declared	Not declared	SPF+AP12	SPF
Piglets weaned/sow/year ²	30.7	27.1	25.4	32.3
1st parity litters (%) ²	20	22	21	23
Recruitment of gilts	Purchase	Own production	Purchase	Own production
Sow feed ³	Wet/ Home made	Wet/ Home made	Wet/ Home made	Wet/ Factory made

¹: Specific Pathogen Free - disease surveillance programme. Danish herds can participate in this programme which registers presence of certain infectious diseases, including PRRS, *M. hyopneumoniae*, *A. pleuropneumoniae*, *P. multocida* tox+ and *B. hyodysenteriae*. ²: Average values calculated from herd-registrations made in a 3-month-period prior to investigation. ³: Feed type used in farrowing period.

elongated with an increased number of mitotic figures. Disruptions in normal epithelial architecture with preserved integrity of the epithelium were recorded as mild epithelial lesions. Diffuse necrotic changes in the epithelium and lamina propria were recorded as mucosal necrosis.

The presence of parasites (*Cryptosporidium* spp, *Giardia* spp, *Cystoisospora suis* and *Strongyloides ransomi*) was examined using standard diagnostic criteria.

Bacteriology

Sections of jejunum and colon were aerobically cultured for *E. coli*. Parallel culturing on Drigalski (in house selective and indicative medium for coliforms) and blood agar plates (Columbia agar (Oxoid) supplemented with 5% calf blood) was performed. Plates were incubated for 24 hours at 37°C. Piglets were considered *E. coli* positive if any growth of haemolytic colonies or moderate/ massive growth of non-haemolytic colonies was seen in any section of intestine. Serotyping of *E. coli* was performed - using one isolate per piglet - by agglutination with monovalent O-antisera (O8, O45, O64, O138, O139, O141, O149 and O157, Statens Serum Institut, Copenhagen, Denmark) [18] and real-time PCR was performed for detection of virulence factor genes F4, F5, F6, F18, F41, STa, STb, LT and VT2e [18]. If no agglutination with antisera was seen, the isolate was designated non-typeable. If agglutination occurred in all pools, the isolate was considered to be O-rough.

Culturing of CP was carried out using Columbia agar (Oxoid) supplemented with 5% calf blood and polymyxin incubated anaerobically for 24 hours at 37°C. Colonies were verified using Tryptose-Sulfite-Cycloserine agar (Oxoid). Piglets were considered culture positive if moderate/ massive growth was observed in any section of intestine. Typing of suspected samples was performed by PCR [19] on a pool of four isolates having characteristic colony morphology on Columbia agar (shiny, grey, double-haemolytic colonies). Culturing of CD was performed using Cycloserine Cefoxitin Fructose Agar, incubated anaerobically for 48 hours at 37°C. Piglets were considered positive if yellow colonies with a characteristic horse-stable odour were detected in any section of intestine.

Virology

Contents of jejunum were examined for rotavirus group A by an enzyme immunoassay (ProSpectT[®] Rotavirus) according to the manufacturer's instructions and for coronavirus by a pan-corona RT-PCR assay as previously described [20].

Statistics

Positive associations between diarrhoea and microbiological and pathological findings were evaluated using

one-sided Fisher's exact tests ($\alpha=0.05$). When considered relevant, associations between histopathology and necropsy were also evaluated by Fisher's exact tests ($\alpha=0.05$). Since the piglets originated from four different herds, associations within herds were also assessed.

Ethical approval

The present study was not subject to ethical approval as Danish laws do not require ethical approval for studies not involving different treatment groups or blood testing. The study only involved procedures normally used for routine diagnostics.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

All authors contributed to the design of the study. Inclusion of herds, clinical examination in the herds and selection of piglets was done by HK. Necropsy was performed by SH and histological examinations were performed by BJ. Culturing of *Clostridium difficile* was done by BK and virulence gene determination of *E. coli* isolates by ØA. LEL did the pan-corona RT-PCR assays. HK conducted the statistical analysis. All authors participated in drafting the manuscript and proofreading of the final manuscript.

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7. General discussion

In this section the aspects of methodology, not addressed in the manuscripts, are discussed. Furthermore, a general summary of discussions of all results described in this thesis, including Manuscripts I-IV are presented.

7.1. Discussion of the methods used in the project

Microscopic investigation

The main objective of the microscopic investigation performed in this PhD project was to describe pathological changes in the intestinal mucosa developed in the course of NNPDS, suspected to be an emerging syndrome in newborn piglets. All microscopic investigations were performed by the author of this thesis and included traditional light microscopic histopathological examination supported by morphometry and immunohistochemistry, as well as fluorescence in situ hybridization for detection of potentially pathogenic bacteria.

As the etiology of NNPDS was unknown prior to this study, the overall histopathological investigation was mainly focused on determination whether the intestinal lesions, if any, resemble those seen in other, etiologically well-established diarrheic conditions. The histopathological changes in the intestinal tissue were determined based on the knowledge of 'normal' histological appearance in healthy individuals. This was performed blindly to the diarrheic status of the piglets, bearing in mind that the non-diarrheic piglets were collected from the same herds and could have also been affected by the syndrome [Kongsted *et al.*, 2013, pp. 121-131]. Villus atrophy and crypt hyperplasia were determined based on the morphological alterations of the normal villus and crypt architecture including morphology of the intestinal epithelium and cellularity of the lamina propria [88]. However, the microscopic appearance of the mucosa during diseases that cause villus atrophy depends on the degree of the epithelial cell loss and regeneration that has occurred by the time the intestinal tissue is examined. In the early phase of cell loss, before the regeneration from the crypt occurs, the villi may be shorter than normal without showing obvious changes in the morphology of the epithelium or the lamina propria. Similarly, as the regeneration occurs, the villi become progressively longer and covered with the well-differentiate epithelium [88]. Therefore, the scoring of villus atrophy applied in this study could lead to misclassification due to false negative results. In order to provide more reliable results, a morphometric measurement has been performed (Manuscript I).

Morphometry is a more sensitive method compared to descriptive observations based on visual assessment of the tissue morphology. However, there are several issues of concern regarding using morphometry for studying the intestinal mucosa. First of all, accurate morphometric assessment requires well-oriented tissue, in which complete villus-crypt units cut longitudinally can be distinguished. This is however very difficult to obtain as the intestinal mucosa, especially in the region of villi, is not rigid and very often the section is cut tangentially which may lead to false interpretation if such sections are measured [258]. To overcome this obstacle, we decided to measure only the highest villi present in the transverse section and we increased a number of investigated sections by preparing an additional slide. However, depending on the availability of well-oriented sections, the number of villi and crypts measured in each intestinal region differed between the piglets resulting in a wide range of measurements (7-18 measurements of villus height, 10-20 measurements of crypt depth) (Manuscript I). Furthermore, this

investigation was performed on the tissue samples representing each intestinal region of the small intestine, and colon. The jejunal sampling sites have been described as 'mid-jejunum'. As the anatomical distinction of duodenum, jejunum and ileum in newborn piglets is fraught with difficulties and the jejunum represents most of the length of the small intestine, so the chances for obtaining jejunal samples from exactly the same site from all animals were rather small. This could affect the results of comparison of villus height values between the diarrheic and non-diarrheic piglets. Finally, the statistical analyses applied in the morphometric study aimed on determination whether age and duration of diarrhea had impact on mucosal morphometry. Results of these analyses should be interpreted with caution, as the number of piglets having diarrhea for a certain number of days in each age group was relatively low (for example only one 6-day-old piglet having diarrhea for 4 and 5 days).

Alternatively to morphometry, a stereological assessment of the intestinal mucosa could be a more adequate and sufficient approach for studying alterations in mucosal morphology. Stereology has been widely performed in research on the intestinal development and functions and this method is used whenever investigation of the intestinal surface area is of special interest [259-261]. In our study, stereological estimation of mucosal surface area would be a better tool for determining the absorption capacity of the mucosa and as such would help better to understand the pathomechanism of NNPDS. However, in order to obtain unbiased results, stereology requires systematic random sampling strategy [262]. This could not be applied in this study, as the sampling procedures were design to achieve all objectives of the overall NNPDS project and the whole length of the intestine was not available for morphometry.

Orientation of the specimen is also essential for accurate assessment of apoptosis in the intestinal tissue. As the primary site of epithelial apoptosis in normal condition is the villus tip, lack of well-oriented villi in tangentially cut section could result in underestimation of apoptotic cells visualized by immunohistochemical staining (Manuscript II). Taking into consideration these technical difficulties when analyzing the formalin fixed paraffin embedded samples, we decided to assess apoptosis in a descriptive manner. Investigation of cell proliferation and apoptosis markers was performed on the tissue samples from the selected piglets from all herds. Selection was based on the presence or absence of the atrophic changes in the mucosa, however, age of the animals was not taken into consideration during selection. It has been shown that during the first days after birth the proliferation of crypt stem cells rapidly increases resulting in increase of total number of the epithelial cells and intensive growth of the intestinal mucosa [47]. As the maturation of the gut occurs, the mitotic index and the ratio between mitotic and apoptotic cells decreases and are comparatively lower in piglets aged 7 days [47, 263]. In order to obtain more reliable results, the investigation of cell proliferation and apoptosis should be further expanded to include more animals and consider changes in epithelial turnover ratios depending on age.

Fluorescence in situ hybridization technique is very useful for investigation of certain bacteria in a tissue sample and their association with the intestinal mucosa, but the quantification of bacteria in a given sample by counting fluorescence signals is not precise. Several studies have used this method to quantify specific bacterial divisions in fecal samples [264-266]. However, visual counting of bacteria in the intestinal tissue is difficult due to a large number of bacteria that very often are clustered in groups. This hinders distinction of a single bacterial cell. In this study, the amounts of bacteria present in the tissue were assessed in semi-quantitative manner, using a scale of small, moderate and large amount (Manuscript III). The results of this

investigation should be interpreted carefully as the differences between the piglets could be due to the degree of entrapment of bacteria between the villi. Precise enumeration of bacteria in a given tissue sample could be investigated by the means of other methods that have been reviewed [267]. However, this was of a lesser interest in relation to this PhD project as the molecular methods such as a high-throughput qPCR and next generation sequencing aiming on quantitative analyses of the intestinal microbiota, including the potentially pathogenic bacteria investigated in this study, were implemented in the other part of the NNPDS project (PhD; intestinal microbiota) [268].

Animal infection model (Manuscript IV)

The results of the experimental study confirmed the hypothesis on an infectious nature of NNPDS. This model can be a useful tool for future investigations on the pathogenesis of NNPDS and establishment of prevention methods. However, there are few issues of concern regarding the study design that should be avoided when using this model in the future. The main issue of concern in this experiment was lack of true control animals. The inoculated and non-inoculated piglets were kept together which most likely resulted in transmission of the infection to the non-inoculated individuals. This could lead to misinterpretation of the results and should be avoided when using this model in the future. Another issue of concern was an unknown virulence of the inoculum derived from naturally infected animals. Not all inoculated piglets within the litter developed diarrhea in our experiment. This could be due to inadequate infectious dose, however biological variability among individuals could also contribute to these results. Similar observations were reported from a recent study on Human Norovirus pathogenesis in a gnotobiotic pig model and lack of the symptoms in some of the infected animals was speculated to be a result of the genetic variability [269]. To increase infection and diarrhea in our model, further serial pig passages of the inoculum and assessment of other variables affecting the clinical outcome (e.g. immune status of the piglets) may be necessary.

In order to determine whether NNPDS is of a viral or bacterial etiology, we used a filtration technique for preparation of inocula. No diarrhea was reproduced when the filtrated, deprived of bacteria inocula were administered to the piglets, indicating that bacterial agents are necessary to induce NNPDS in experimental conditions. However, if viral agents were to be involved in the pathogenesis of NNPDS, the successful reproduction of the symptoms in these piglets could have been influenced by infectious dose of the virus in the filtrate. It is therefore possible that the potential viral agent was present in the filtrate but its amount was insufficient to cause the symptoms. Therefore the results of this study alone cannot definitely exclude a potential viral etiology of NNPDS. In order to investigate a viral involvement in the syndrome, a number of assays such as Real-Time PCR (RT PCR), Transmission Electron Microscopy (TEM), microarray and Next Generation de novo Sequencing (de novo NGS) were implemented in the other part of the NNPDS project (Larsen *et al.*, 2014; unpublished data). Those studies were performed on the samples (intestinal tissue, intestinal contents and rectal swabs) collected from the diarrheic and non-diarrheic piglets from the field study. Coronaviruses, including Porcine Epidemic Diarrhea Virus (PEDV), Porcine Respiratory Coronavirus (PRCV) and Transmissible Gastroenteritis Virus (TGE), astrovirus, rotavirus A, rotavirus C, norovirus, sapovirus, enterovirus, parechovirus, saffoldvirus, cosavirus, aichovirus and klassevirus were investigated by RT PCR. Additionally, ileal tissue samples from selected piglets with pronounced villous atrophy were investigated by TEM, microarray and de novo NGS for presence of unspecific viruses. No evidence of a viral involvement was shown in those studies (Larsen *et al.*, 2014; unpublished data).

7.2. Histopathological changes characteristic for NNPDS

The histopathological, morphometrical and immunohistochemical investigations performed on the tissue specimens from 101 piglets collected from four herds affected by NNPDS showed that the diarrheic piglets differed from non-diarrheic individuals in the intestinal morphology. In general, the villi in the jejunum and ileum were significantly shorter and the crypts of Lieberkühn were significantly deeper in the diarrheic piglets compared to the piglets without clinical symptoms (Manuscript I). Mild to moderate villus atrophy in the jejunum and ileum, characterized by deformation of the villi, flattening of the villus epithelium and increased cellularity of the lamina propria were the most frequent microscopic lesions and was seen in 63% of the diarrheic piglets (Chapter 4.1.). Villous atrophy was frequently associated with intestinal crypt hyperplasia resulting from enlargement of the proliferative compartment. Enhanced mitotic activity of the crypt cell was most likely a response to the cell damage in the villus epithelium (Manuscript II). Cell damage was not frequently observed in the histological sections. However, mild epithelial lesions at the villus tips and luminal surface in the colon including flattening, desquamation, erosions and necrosis of the enterocytes were present in some piglets and were also associated with diarrhea (Chapter 4.1). These two features- villus atrophy with crypt hyperplasia in the jejunum and ileum, and mild epithelial lesions can be regarded as characteristic for NNPDS. Other histological changes such as neutrophil infiltration, edema, congestion, hemorrhages in the lamina propria and necrosis of the mucosa were developed in the piglets, however, their occurrence was occasional. These additional changes seemed to be related to the presence of bacterial agents and correlated with severity of the symptoms (Chapter 5.1, Manuscripts III and IV). The lesions seen in the piglets suggest that pathomechanism of NNPDS is related to disrupted integrity of the epithelial barrier due to epithelial cell loss and can be classified as malabsorptive diarrhea as a result of reduced mucosal surface area available for absorption [88]. Diarrhea in the course of NNPDS seems to be mainly related to dysfunction of the small intestine; however, the presence of the epithelial lesions and crypt elongation in the colon suggest that also the large intestine can be involved (Chapter 4.1., Manuscript I).

Histological lesions were occasionally present in some of the non-diarrheic piglets in this study. Villus atrophy associated with crypt hyperplasia were seen in 12% of the non-diarrheic piglets, however this lesion was confined to either jejunum (n=1) or ileum (n=5) (Chapter 4.1). As the non-diarrheic piglets were collected from the same herds as the diarrheic ones, it cannot be excluded that they were in the incubation phase of NNPDS. Individual differences in immune status of the piglets could also be a factor affecting the clinical course of diarrhea. It is possible that some piglets were able to resist NNPDS without having the symptoms. Accordingly, lack of the histological lesions in some of the animals with diarrhea is probably a result of selection for euthanasia at different stages of the syndrome. As shown by the immunohistochemical study, the epithelial turnover was enhanced in the diarrheic piglets (Manuscript II). Increased proliferation of the enterocytes located in the intestinal crypts was more likely directed towards epithelial repair process which enables rebuilding of normal mucosal architecture within a few days after the injury [88]. The morphometric study performed on the piglets showed that the villus height and crypt depth values had tendency to return towards normal values with diarrhea days (Manuscript I). This finding suggests that villus atrophy developed in piglets suffering from NNPDS was reversible. Therefore, it is possible that recovery from the morphological changes occurred prior to restoration of enterocyte functions and this can explain the lack of the histopathological changes in some of the diarrheic piglets.

However, it cannot be ruled out that other pathomechanisms, not associated with visible morphological changes, i.e. increased intestinal permeability due to disruption of the tight junction functions or secretory diarrhea, were involved in development of NNPDS in these piglets.

In general, the histomorphological changes developed in the intestinal mucosa in the course of NNPDS were not specific and neither could indicate the exact etiology of NNPDS nor distinguish this syndrome from other, classical form of neonatal diarrhea. Villus atrophy with enlargement of crypt proliferative compartment is a common lesion in many diarrheic diseases and is especially associated with viral and parasitic diarrheal conditions [94, 95, 128, 137, 151, 160, 167]. However, the histopathological investigation on the HE stained intestinal tissue sections showed no presence of the parasites in the piglets and the microbiological tests performed in other parts of the NNPDS project showed no evidence of involvement of viral agents.

7.3. Possible etiology of NNPDS

The hypothesis on an infectious nature of NNPDS was confirmed by successful reproduction of the clinical symptoms and histological lesions by per oral inoculation with intestinal homogenate derived from naturally infected animals (Manuscript IV). However, the exact pathogen causing the infection could not be established in the studies described in this PhD thesis. Although the histopathological changes developed in the course of NNPDS resembled viral diarrhea, the results of the microbiological tests performed in other parts of the NNPDS project could not establish a viral etiology of the syndrome. Results of conventional pan-corona assay and RT-PCR tests for detection of coronaviruses were negative in all piglets (Larsen LE, *et al.*, 2014, unpublished data). These results were as expected, since TGE and PED have not been diagnosed in Denmark. As shown by Kongsted *et al.*, 2013, only one diarrheic piglet was positive for rotavirus A by a commercial ELISA test. RT-PCR assays detected further seven piglets (7/76) positive for rotavirus A (Larsen LE, *et al.*, 2014, unpublished data). In the experimental infection performed in this study, nine piglets were positive for rotavirus A (Manuscript IV). This relatively low prevalence of positive piglets indicates that rotavirus A was not a significant problem in NNPDS. Among other viruses investigated in the virological study, only kobuvirus and teschovirus have been detected by de novo NGS in the piglets affected by NNPDS. However, the role of these viruses in pathogenesis of diarrhea is unknown and these tests were performed on a low number of selected tissue samples, thus further studies are needed to conclude on the involvement of these viruses in NNPDS (Larsen LE, *et al.*, 2014, unpublished data).

Based on the results of the investigations performed in this PhD project, there is evidence that bacterial agents were involved in the pathogenesis of the syndrome. Firstly, as shown in Manuscript IV, experimental induction of diarrhea was not possible when the intestinal tissue filtrates were administered to the piglets, which favors the hypothesis on the bacterial, rather than viral etiology of NNPDS. Secondly, FISH investigation performed on the fixed tissue samples from 101 piglets revealed colonization of the small intestinal mucosa by adherent bacteria in 37% of the diarrheic and 14% of the non-diarrheic piglets (Manuscript III). FISH with the specific probes identified these adherent bacteria as *E. coli* and *Enterococcus* spp. and in most cases these bacteria colonized the mucosa simultaneously. The presence of adherent *E. coli* was seen in 33% of the diarrheic and 14% of the diarrheic piglets and was associated with villous atrophy and neutrophil infiltration regardless the diarrheic status. Adherent enterococci were present in 45% of the diarrheic and 8% of the non-diarrheic piglets. The presence of enterococci in the small intestinal

mucosa was associated with villus atrophy and the adherence of these bacteria was associated with epithelial lesions. These findings suggest that simultaneous colonization of the mucosa by adherent *E. coli* and *Enterococcus* spp. may play role in the pathogenesis of NNPDS. Additionally, FISH investigation of the intestinal tissue from the piglets with experimentally induced NNPDS (litter 2) also showed that these adherent bacteria could be involved in the syndrome (Chapter 5.1.2). In the majority of the piglets with adherent *E. coli* and *Enterococcus* spp. in both natural and experimental infection the microscopic lesions seen in the mucosa were more pronounced compared to the piglets in which these bacteria were not detected (Chapter 4.1. and Manuscript IV). Moreover, the piglets from litter 2 with experimentally induced NNPDS showed severe clinical symptoms including poor body condition and dehydration. This suggests that adherent *E. coli* and *Enterococcus* spp. worsen the clinical and pathological manifestation of NNPDS. As adherent enterococci and *E. coli* were mostly detected in the piglets from one herd (Herd 2), whereas in the other herds these bacteria were seen only sporadically, it can be concluded that herd-related factors are important in the pathogenesis of NNPDS and may influence composition of intestinal microbiota, hence the clinical outcome of diarrhea.

Enteroadherent *E. durans* has been shown to cause diarrhea in suckling animals including piglets [179-184]. However, FISH with the specific probe for *E. durans* performed on the tissue samples from selected piglets positive for enterococci showed negative results (Chapter 5.1.2). Identification of enterococci found in the piglets in this study was further continued by traditional culturing of the intestinal tissue from selected piglets positive for adherent enterococci by FISH (n=20). *E. hirae* was identified in majority of these piglets (n=16) (personal communications, Jensen TK, National Veterinary Institute, Technical University of Denmark). Recently, enteroadherent *E. hirae* was demonstrated in the piglets suffering from neonatal diarrhea resembling NNPDS in Sweden and its presence was associated with villus atrophy, mild epithelial lesions and apoptosis of the epithelial cells [188]. These findings correspond well with our results which showed association between enterococci and histological lesions. This supports our conclusions on the involvement of these bacteria in neonatal porcine diarrhea. Similarly, FISH investigation showed evidence that adherent *E. coli* were associated with NNPDS. Although enterotoxigenic *E. coli* that adhere to the epithelial cells and releases toxins is known to be one of the most common causes of neonatal diarrhea during the first week of life, it seems not to play any important role in relation to NNPDS [Kongsted, *et al*, pp. 121-131]. It has been suggested that non-ETEC strains of *E. coli* with ability to adhere to the intestinal mucosa (i.e. enteroaggregative *E. coli* and attaching and effacing *E. coli*) may have a potential pathogenic role in relation to porcine diarrhea [224, 228, 230]. Recently, it has been speculated that a heat stable enterotoxin 1 (EAST1) and adhesin involved in diffuse adherence AIDA-1 may be implicated in pathogenicity of these adherent *E. coli* [226, 236, 238]. Investigation on the *E. coli* virulence factors from the *E. coli* isolates found in the piglets was carried out in the other part of the NNPDS project. That study revealed that some piglets were positive for EAST1 and AIDA-1 genes (personal communications, Angen Ø, Norwegian Veterinary Institute, Norway). However, the exact role of these factors in neonatal diarrhea has not yet been established and remains to be clarified.

There was no clear indication for the involvement of clostridial species in NNPDS upon histopathological and FISH investigations. *C. perfringens* type A has, in recent years, emerged as a potential pathogen and is frequently detected in piglets with diarrhea [147, 202, 203]. In this study, FISH investigation with the specific probe detected *C. perfringens* in the majority of the diarrheic and non-diarrheic piglets (73% vs. 78%) and the amounts of these bacteria in the intestinal mucosa did not differ significantly between the

piglets (Manuscript III). However, histological lesions associated with *C. perfringens* infection such as necrotizing enteritis were seen in one diarrheic piglet that was positive for *C. perfringens* type C in culturing [Kongsted *et al*, 2013, pp.121-131]. Additionally, FISH study has shown that in 20% of the diarrheic and 30% of the non-diarrheic piglets these bacteria were present within the mucus layer and in a direct contact with the epithelium, which may suggest their potential ability to affect the epithelial barrier (Manuscript III).

C. difficile is in some regions regarded as a common agent involved in neonatal diarrhea in piglets [147, 208-211]. The primary site of the infection with this bacterium is colon [147]. In this study, FISH performed with the specific probe detected small amounts of *C. difficile* in the colon of 65% of the diarrheic and 70% of the non-diarrheic piglets but there was no correlation between the presence of bacteria and epithelial lesions in the colon. (Manuscript III). As the pathogenicity of clostridial infection is believed to be associated with the toxins produced by these bacteria, detection of clostridia in the tissue alone is insufficient to conclude whether they were involved in the syndrome. Further investigations are needed to clarify the role of clostridia in the pathogenesis of NNPDs. Studies on the clostridial toxins in the piglets affected by NNPDs have been implemented in the other part of the NNPDs project. The toxins (α and β 2) were investigated by Elisa tests on the intestinal contents from the piglets investigated in the field study, however, the results of this investigation were inconclusive (personal communications, Kokotovic B, National Veterinary Institute, Technical University of Denmark, Denmark).

8. Conclusions and perspectives

The studies performed in this PhD project have contributed to the knowledge of NNPDS. Based on the results presented in this thesis it can be concluded that NNPDS is an infectious syndrome and its pathomechanism is related to malabsorptive diarrhea due to villus atrophy, secondary to epithelial cell damage. Histological lesions developed during the course of NNPDS are non-specific and include villus atrophy of various degrees with enlargement of crypt proliferative compartment in the jejunum and ileum and occasionally mild epithelial lesions in the entire intestine. Inflammation and necrotizing enteritis develop seldom and are associated with complication by bacterial agents. However, it is difficult to determine whether malabsorptive diarrhea is an exclusive pathomechanism of NNPDS. It is possible that the lack of the histological lesions in some diarrheic piglets was related to other types of diarrhea. In fact, in most of the infectious diarrheic diseases multiple mechanisms can be distinguished [111, 113]. Therefore, it would be interesting to investigate other aspects of intestinal barrier integrity in the affected animals, e.g. tight junction functions. For instance, measurement of intestinal permeability could be a useful approach to assess the loss of mucosal integrity and absorptive area. In order to further characterize the pathological changes, electron microscopy could be applied on the intestinal tissue samples. Investigation on the ultrastructural alterations in the damaged enterocytes could contribute to better understanding of the pathogenesis of the syndrome. Electron microscopy would also be a good method to support study on the eventual viral etiology of NNPDS.

Experimental study performed as a part of this PhD project showed that the clinical symptoms and histological lesions characteristic of NNPDS can be reproduced by inoculation of newborn piglets with homogenized intestinal tissue derived from naturally infected animals. These results confirm the hypothesis on the infectious nature of NNPDS. However, so far, the exact agent(s) has not been established by the investigations undertaken in the overall NNPDS project and requires further studies. Although pathologically NNPDS does not differ significantly from other classical forms of infectious diarrhea in neonatal pigs, the lack of well-established etiology favors the hypothesis that NNPDS is a new syndrome. Recent studies on the etiological causes of neonatal diarrhea in other countries also showed a general tendency to a relative decrease in diagnoses of ETEC, TGE and necrotizing enteritis caused by *C. perfringens* type C and an increase in diarrhea cases with no identified etiological agent or attributed to new emerging pathogens such as *C. difficile* and *C. perfringens* type A [211, 270]. Therefore, further investigations on the etiology and involvement of the emerging pathogens in NNPDS could bring benefits to global pig production. Based on the results of FISH investigation performed in this PhD project, it can be concluded that simultaneous colonization of the intestinal mucosa by adherent, non-enterotoxigenic *E. coli* and *Enterococcus* spp. is involved in the pathogenesis of NNPDS and may enhance severity of the symptoms. Regarding enterococci, *E. hirae* may be a potential candidate possibly involved in the pathogenesis of the syndrome. However, the pathogenicity of the adherent bacteria found in this study and their interactions remain to be investigated. Experimental infection of newborn piglets with adherent non-ETEC *E. coli* and *E. hirae* found in the diarrheic piglets would be the best method to clarify the role of these bacteria as a potential cause of NNPDS. Furthermore, FISH investigation performed in this PhD project showed no association between the occurrence of *C. perfringens* type A and *C. difficile*. *C. perfringens* type A has in recent years gained a particular importance as a cause of neonatal diarrhea in piglets, however, the pathogenesis of the infection with this bacteria is not well-understood. Detection of $\beta 2$ toxin, which is

believed to be linked to *C. perfringens* type A pathogenicity, does not have diagnostic relevance in itself as the toxigenic strains of *C. perfringens* type A can be found in both healthy and diarrheic piglets [147, 270]. A better approach could be detection of the toxin in clinical samples in situ and demonstration of its association with the pathological changes in the intestinal mucosa. This can be possible by the means of immunohistochemical staining of the clinical samples with anti- β 2-toxin antibody [271].

Currently, large scale epidemiological and pathological investigations on NNPDS in Denmark are scheduled with the aim to determine whether the diarrheic piglets from herds affected by the syndrome can be pathologically and microbiologically distinguished from the diarrheic piglets from healthy herds. These investigations will include gross pathology, detection of rotavirus, *E. coli* (including *E. coli* virulence factors), *C. perfringens* and *C. difficile* (including clostridial toxins). Additionally, to date, the investigation on the intestinal microbiota in the piglets affected by NNPDS has been carried out by use of the Gut Microbiotassay [268]. If the pathogenesis of the syndrome is related to an unbalanced composition of the intestinal microbiota, this new molecular approach will promisingly address this aspect. In perspectives, the animal model of infection established in the study presented in this thesis can be a useful tool for further investigations of the pathogenesis of NNPDS. As mentioned above, experimental infection of newborn piglets with adherent *E. coli* and enterococci could determine their pathogenicity. Similarly, experimental infection with new viruses detected in the piglets (kobuvirus and teschovirus) could be helpful to elucidate their role in the syndrome. Finally, despite the lack of successful diagnosis of the exact etiology, the animal model of NNPDS can also be used for establishment of treatment strategies, e.g. testing the effect of probiotics, prebiotics and other strategies alternative to antimicrobial agents on diarrhea incidence and intestinal microbiology and pathology. Establishment of treatment could benefit the Danish swine industry in economic and animal welfare aspects.

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Appendix A

Immunohistochemical staining for detection of cell proliferation (Ki-67) and apoptosis (cleaved caspase-3)

Reagents:

- Primary antibodies:
 - Monoclonal Mouse Anti-Human Ki-67 Antigen Clone MIB-1 (DAKO, Code nr. M724029-2)
 - Cleaved Caspase-3 (Asp175) Antibody #9661 (Cell Signaling Technology, No. 9661S, BioNordika Denmark A/S Produktionsvej 26 DK-2600 Glostrup)
- Envision™ G/2 Doublestain System, Rabbit/Mouse (DAB+/Permanent Red), (DAKO, code nr. K5361)
- Tris buffered saline- TBS (50 mM Tris - Sigma, 150 mM NaCl, pH 7.6)
- Mayer's hematoxylin
- Mounting medium: glycergel (Dako, Glostrup, Denmark).
- TRIS/EDTA buffer (10 mM Tris-Sigma, 1 mM EDTA Titriplex III, pH 9,0; Merck, Darmstadt, Germany)

Protocol:

1. Deparaffinization and dehydration
Place the slides in a rack, and perform the following washes:
Xylene: 2 min
Xylene: 2 min
99% Ethanol: 2 min
99% Ethanol: 2 min
96% Ethanol: 2 min
Running cold tap water to rinse
2. Heat-Induced Epitope Retrieval (HIER)
Place the slides in a rack in TRIS/EDTA buffer, pH 9.0. Heat the slides in a microwave oven 1x6 min 800W followed by 2x5 min 600W. Leave the slides in the buffer for 40 min in room temp.
3. Wash 2x5 min in TBS
4. Dual endogen enzyme block for 3 min
5. Wash 2x5 min in TBS
6. Primary antibody:
 - a. Monoclonal Mouse Anti-Human Ki-67 (diluted 1:100 in TBS). Incubate for 30 min (room temp.)
 - b. Cleaved Caspase-3 (diluted 1:1000 in TBS). Incubate over the night in the fridge
7. Wash 2x5 min in TBS
8. Secondary antibody Polymer/HRP. Incubate for 30 min (room temp.)
9. Wash 2x5 min in TBS
10. DAB+Working solution. Incubate for 10 min (protect from direct light)
11. Wash with distilled/deionized water and in TBS
12. Wash with distilled/deionized water and leave in water bath for 5 min
13. Counterstaining with Mayer's hematoxylin diluted 1:2 for 20 sec
14. Wash the slides in water and mount with the glycergel

Appendix B

Fluorescence in situ hybridization (FISH) for potentially pathogenic bacteria

1. Sequences of the probes (Eurofins MWG Operon, Ebersberg, Germany).

Target bacteria	Name of the probe	Target sequence (5'-3')	Target region of rRNA	References
<i>Domain bacteria</i>	S-D-Eub-0338	GCTGCCTCCCGTAGGAGT	16S	[272]
<i>Escherichia coli</i>	S-S-E.coli-1161	GCATAAGCGTCGCTGCCG	23S	[273]
<i>Enterococcus spp.</i>	S-G-Enteroco-184	CAAATCAAAACCATGCGG	16S	[274]
<i>Clostridium perfringens</i>	S-S-C.perfring-1	TGGTTGAATGATGATGCC	16S	[275]
<i>Clostridium difficile</i>	S-S-C.diff-193	TGTACTGGCTCACCTTTG	16S	[276]
<i>Enterococcus durans</i>	E. durans	CTTACTCGTGTAGACAGA	23S	[277]

2. In-house protocol for FISH on formalin-fixed paraffin embedded tissue

Samples are mostly formalin-fixed and paraffin embedded tissue sections, app. 3 µm thick and dry-mounted on microscope slides.

Before hybridization paraffin must be removed by washing the slide in: xylene (2 x 2 min), 99 % ethanol (1 x 2 min) and 96 % ethanol(1 x 2 min) before being transferred to 96 % ethanol (slides can be stored in ethanol for up to one day without problems).

Equipment and reagents used for hybridization:

- Shandon hybridization rack consisting of a bottom container, a top rack with 10 slots for hybridization chambers and a lid

- Shandon Disposable Coverplates for assembling hybridization chambers (see fig 1 & 2)

- Buffers: - In situ Hybridization Buffer: 1M Tris (pH 7.2) - 50 ml
5M NaCl - 90 ml
10% SDS - 5 ml
H₂O to 500 ml

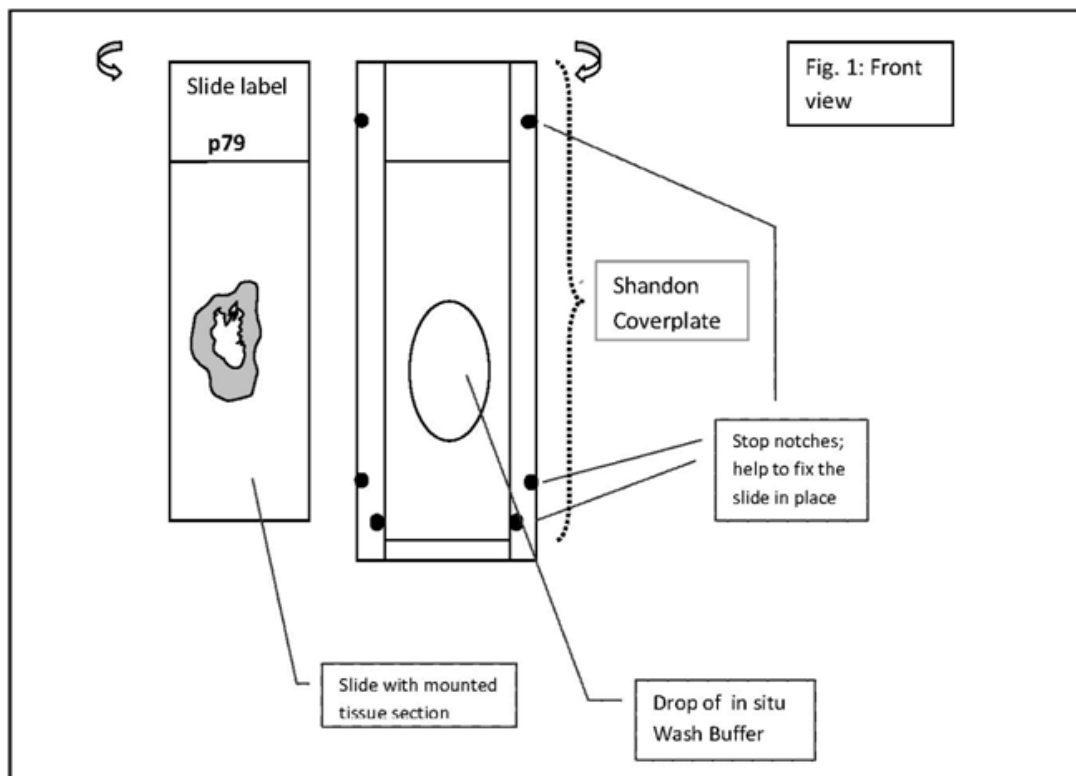
- In situ Wash Buffer: 1M Tris (pH 7.2) - 50 ml
5M NaCl - 90 ml
H₂O to 500 ml

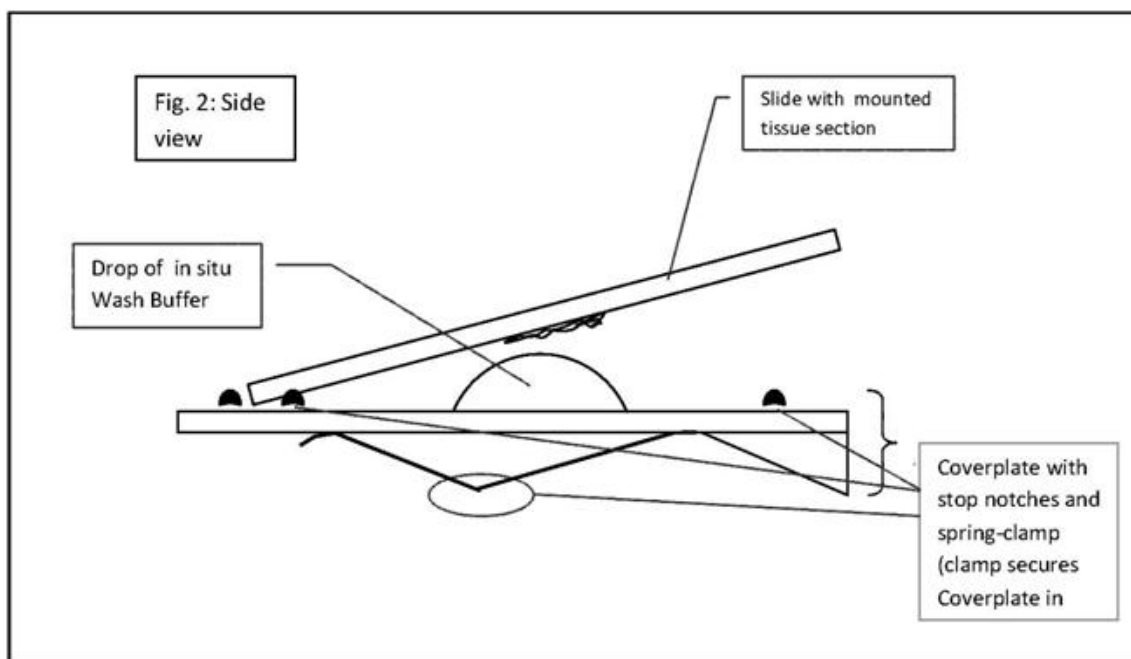
- Fluorescent probes: amount of probe necessary for hybridization varies (see below)

Procedure: Day 1

1. Remove microscope slide from ethanol and let it air-dry on a clean paper napkin.
2. In order to be able to calculate how much hybridization mix will be necessary note probe/probes combination(s) which are to be used and number of slides per each probe/probes combination. The probes are registered in our probe list under a serial number preceded by a small letter p (for “probe”). The number of probe to be used should be written in advance (it is usually done in histology lab) on the slide’s label; e.g. p79.
3. Open the Shandon rack by lifting the top rack. Pour some few ml. deionized water in the bottom container (a mark inside shows the max vol. water might be filled to). Replace the top rack.
4. Place the rack lid bottom up on the bench. Lay the Coverplate horizontally across the rack lid, front side up. Place few drops of in situ Wash Buffer on the Coverplate. (Fig. 1 and 2)
5. Place the tissue slide on the Coverplate, tissue sample facing the front of the Coverplate. In order to avoid air bubbles moisten first the specimen with In Situ washing buffer (Fig. 1 and 2)

The slide should be placed within the 6 stop notches on the Coverplate (fig. 1). A hybridization chamber of about 80 μ l is thereby formed between the Coverplate and the slide.





Place the hybridization chamber upright in the Shandon rack chamber slot so that the rectangular stop notch in the slot fits exactly ("clicks" in place) into the rectangular hole in the middle of the spring-clamp.

6. Prepare separate probe mix for each probe/probe combination used for the hybridization. Calculate with one extra slide per each mix. You will need 100 μl mix per slide. The probe concentration in mix is to be 5 $\mu\text{g}/\mu\text{l}$ for each probe.

Calculation of volume of stock solution of probe: (V-volume; C – concentration; n – number of slides)

$$V_{\text{probe}} = \frac{V_{\text{buffer per 1 slide}} \times (n_{\text{slide}} + 1) \times C_{\text{probe in mix}}}{C_{\text{probe in stock}}}$$

or

$$\mu\text{l}_{\text{probe}} = \frac{100 \mu\text{l}_{\text{buffer}} \times (\text{amount of sample} + 1) \times 5 \text{ ng}/\mu\text{l}}{\text{Conc. (in ng}/\mu\text{l}) \text{ of probe in stock}}$$

Stock concentrations of probes are registered in the probe list (H:\Mikrobio\Genteknologi\Seurat\In-Situ\In-Situ prober or in a printed copy (the orange folder) in laboratory 3.06

7. Apply 100 μ l of the right hybridization mix to the hybridization chamber's top well (Fig. 3. step 1 to 3).

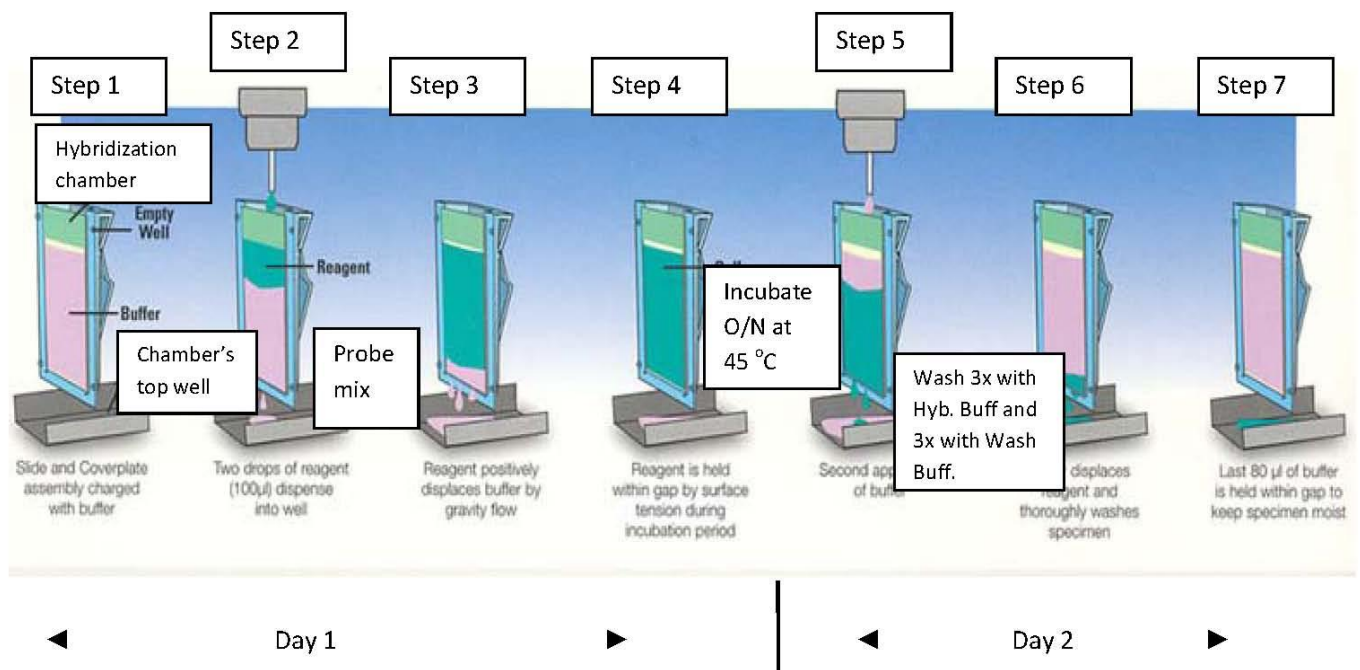


Fig 3. **Day 1:** step 1 – 3; **Over Night:** step 4; **Day 2:** step 5-7 (Shandon rack is not shown)

8. Place the lid on the Shandon rack, wrap it in tinfoil and incubate over night at 45 $^{\circ}$ C (incubator in laboratory 3.06). Make sure that you have enough of buffers for the washing procedure. You will need 10 to 12 ml of each buffer per slide. Place the buffers as well O/N in the incubator in order to equilibrate them to the washing temperature of 45 $^{\circ}$ C.

Day 2

9. Remove the tinfoil and the lid from the rack and place it back in the incubator. Apply warm (45 $^{\circ}$ C) Hybridization Buffer (use squirt flask) to the hybridization chamber's top well (Fig. 3, step 5-7). Let the buffer run through and repeat the procedure 2 times. Do the same with the Wash Buffer.

10. Fill a Coplin Jar with MilliQ water, remove the slides from Coverplates place them in the jar and leave in 1 - 2 min at room temperature.

11. Remove the slides from the jar and place them in the incubator on a clean lab napkin or in an open slide box until they dry. Note! Remember that fluorescent probes are degraded by light – therefore protect them and the slides from light when possible. Hybridized slides can be stored at 5 $^{\circ}$ C for 1 to 2 months usually without problems.